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REGENERATION OF THYMUS GRAFTS

II. EFFECTS ON IMMUNOLOGICAL CAPACITY

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SUMMARY

Mice thymectomized at birth were grafted at 1 week of age with thymus tissue under the kidney capsule. The implants were excised after a period of 1, 2 or 3 weeks and the response of the mice to sheep erythrocytes and to allogeneic skin grafts was tested. Thymectomized mice that had not received thymus implants had twenty times less antibody-plaque-forming cells per million spleen cells than sham-thymectomized controls and failed to reject foreign skin. Some evidence of restoration of immune capacities was obtained in mice bearing for 1 to 2 weeks either normal thymus implants or thymus tissue irradiated *in vitro* with 500 R. By contrast, thymus tissue irradiated with 2000 R failed to influence neonatally thymectomized mice with respect to their immunological capacities. Most thymectomized mice bearing thymus implants, whether normal, irradiated with 500 or 2000 R, had blood lymphocyte levels within the normal range. Cytological analysis of the lymph nodes and spleen of mice bearing normal thymus implants revealed only very few thymus-derived cells but the proportion of these cells was significantly increased after specific immunization. No thymus-derived cells were however detected in the lymphoid tissues of mice bearing irradiated thymus implants, not even in those that were capable of responding to antigenic stimuli. It is concluded that the thymus plays an essential role in inducing the differentiation of immunologically competent cells from non-competent precursors and that this function is dependent on the integrity of the thymus epithelial-reticular cells.

The thymus is essential for the development of populations of cells which are responsible for some types of immunological reactions. In mice, neonatal thymectomy has been associ-

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ated with severe immunological defects (Miller, 1961, 1962a, b, 1963; Good *et al.*, 1962) and thymectomy of the adult has been shown to impair the response to a new antigen given 6 months or more after removal of the thymus (Taylor, 1965; Metcalf, 1965; Miller, 1965a). Adult mice subjected to total body irradiation after thymectomy have failed to recover their capacity for reacting to some antigenic stimuli (Miller, 1962c; Miller, Doak & Cross, 1963; Globerson & Feldman, 1964). It has been demonstrated that thymus grafts can restore the immunological capacities of neonatally thymectomized mice (Miller, 1961, 1962b) and of thymectomized irradiated mice (Miller *et al.*, 1964; Leuchars, Cross & Dukor, 1965). The sequence of histological and cytological events taking place during the regeneration of thymus tissue implanted in a subcutaneous site and under the renal capsule has been studied in detail (Dukor *et al.*, 1965). Initially, necrosis affected all cell types leaving only a thin peripheral rim of viable epithelial-reticular cells and lymphocytes. Regeneration took place by differentiation and multiplication of cells surviving in this rim and normal thymus architecture was restored within 7 days in normal and thymectomized hosts. The dividing cell population in the graft became entirely replaced within 15 days by cells coming in from the host. Thymus tissue heavily irradiated *in vitro* initially consisted only of an epithelial-reticular framework totally devoid of lymphocytes. Typical thymus lymphocytes appeared only after 1–2 weeks and normal thymus architecture was restored between 2 and 3 weeks. At no time were donor-type dividing cells detected in these irradiated grafts: dividing cells could first be scored after 10 days and were always of host-type.

The mechanism by which thymus grafts restore immune capacity in thymectomized hosts is not known. The graft may have to provide lymphocytes which become competent after migrating to lymph nodes and spleen; if this is so the presence of thymus-derived cells in such organs would be expected in animals in which immune capacities have been restored. On the other hand, the graft may have to attract host lymphoid precursor cells to enable them to differentiate in its environment to cells capable of reacting immunologically once they settle in lymph nodes and spleen: if this is the case restoration of immunological capacity would not be expected to occur before host cells could have migrated into the graft, an event which begins between 10 and 13 days after grafting (Dukor *et al.*, 1965). Finally, the graft may simply have to provide a humoral factor which would enable the differentiation of host precursor lymphoid cells to immunologically competent cells. In this case, traffic of cells to and from the graft would not be essential. Evidence for the existence of a humoral factor has already been obtained in studies employing thymus tissue in millipore diffusion chambers (Osoba & Miller, 1963, 1964; Law *et al.*, 1964). In order to study the possible mechanisms by which thymus grafts restore immune capacities experiments were performed on neonatally thymectomized hosts bearing thymus grafts for limited periods. Syngeneic and allogeneic thymus grafts, and thymus tissue irradiated *in vitro* with 500 and 2000 R prior to implantation, were used. The grafts were excised after various intervals and the degree of restoration of immunological capacity in the thymectomized hosts was determined.

MATERIALS AND METHODS

Mice. Mice of the highly inbred strains Ak, C3H and C57Bl and F_1 hybrids between Ak and T6 were used in the experiments. The inbred lines have been maintained for many

generations of brother–sister matings at the Pollards Wood Research Station of the Chester Beatty Research Institute.

Thymectomy. Thymectomy or sham-operation was performed on mice less than 24 hr old by a method identical to that used in this laboratory for adult mice (Miller, 1960). Anaesthesia was induced by refrigeration at —15°C for 5 min. Completeness of thymectomy was checked by routine histological examination of the mediastinal area when the mice were sacrificed.

Thymus grafting. Thymuses were removed aseptically from neonatal donor mice and transferred into cold tissue culture medium '199' (Difco). Recipients aged between 7 and 10 days were lightly anaesthetized with ether and the left kidney was exposed and brought out of the operating wound in such a way that a single, intact, thymus lobe could be pushed into the subcapsular space by means of a very fine forceps through a small incision in the renal capsule. All thymus lobes were grafted within 20 min of their removal from the donors. A sham operation was performed in controls not receiving thymus grafts.

Excision of thymus grafts. Under light ether anaesthesia the left kidney was exposed and brought out and the renal capsule overlying the thymus implant was torn away so that the implant could easily be removed from the surface of the kidney. Grafts were removed from 1, 2 and 3 weeks after implantation as indicated.

Irradiation. A Cobalt 60 source of 100 curies was used without added filtration. Freshly excised thymuses were suspended in cold tissue culture medium '199' and irradiated in a glass tube with doses of 515 or 2060 R at a focal distance of 2·5 cm and a dose rate of 1030 R/min.

Immunization. Sheep erythrocytes in Alsever's solution (Wellcome Research Laboratories, Beckenham, Kent) were washed three times in 0·9% sodium chloride and made up to a 5% suspension for injection. Groups of sham-thymectomized, thymectomized and thymus-grafted mice were injected intraperitoneally with 0·25 ml of this suspension when they were between 28 and 31 days of age.

Antibody-plaque-forming cell detection technique. The procedure developed by Jerne, Nordin & Henry (1963) was closely followed. To 2 ml of 0·7% melted purified Difco Agar was added 0·1 ml containing 1 mg of DEAE-dextran (M.W. $2{\cdot}6 \times 10^6$, Uppsala, Sweden), 0·1 ml of 50% suspension of freshly washed sheep erythrocytes and 0·1 ml of Eagle's tissue culture solution containing from 10^6 to 10^7 viable nucleated spleen cells. The warm cell–agar suspension was mixed rapidly and thoroughly and then layered rapidly onto a 2 mm thick base layer of solidified 1·4% agar previously prepared with sterile Eagle's solution in $3\frac{1}{2}$ in. diameter round Petri dishes. Cell suspensions from each mouse were plated in duplicate. Plates were incubated for 1–$1\frac{1}{2}$ hr at 37°C, then flooded with 3 ml of 1:10 dilution of guinea-pig complement (made up from preserved guinea-pig serum obtained from Wellcome Research Laboratories) and re-incubated for 30 min at 37°C. Antibody-plaques could be clearly seen as zones of haemolysis in the turbid pink background of unlysed erythrocytes. The plaques were counted under the dissecting microscope and the number of antibody-plaque-forming cells per 10^6 spleen cells was recorded.

Skin grafts. Full thickness grafts of skin from C3H and C57Bl mice were performed according to the method of Billingham & Medawar (1951). Seven days after grafting, the protective dressings were removed and the grafts were examined daily for signs of rejection. Skin grafts were generally performed when the mice were 35 days of age.

Cytology. Mice carrying the T6 marker chromosome were killed for cytological examination at various times after thymus and skin grafting. Ninety minutes before killing 0·2 ml of a 0·06% 'Colcemid' (Ciba) solution per 10 g body weight was injected intraperitoneally. Cell suspensions from spleens, axillary lymph nodes and, in some cases, thymic implants were incubated at room temperature in 1% sodium citrate for 15 min, centrifuged very gently, fixed repeatedly in freshly made Carnoy's fluid for several minutes according to the technique of Ford (1963, personal communication). The cells were finally spread by an air-drying procedure and stained with aceto-orcein.

Haematology. Total white cell counts were performed from tail blood when the mice were between 2 and 4 months of age. The total lymphocyte count was determined from the percentage of lymphocytes obtained from a differential white count of 1000 cells. Blood cells were stained with Wright's stain (George T. Gurr, Ltd, London).

Histology. Sections from thymus implants, lymph nodes, spleen and thymus area of sacrificed mice were fixed in Bouin's fluid, cut at 5 μ and stained with haematoxylin and eosin. For staining with methyl green pyronin, the tissues were fixed in Carnoy's fluid.

Statistical analysis. The results were analysed statistically by using Student's *t*-test and the χ^2-test.

RESULTS

Blood lymphocyte levels

Sham-thymectomized (Ak × T6)F_1 mice aged over 2 months had lymphocyte levels above 7000/mm^3 whereas 2–4-month-old mice of the same strain thymectomized at birth all showed levels below 7000 and generally below 5000/mm^3. Implantation of normal T6 or C3H thymus tissue restored lymphocyte levels to normal in neonatally thymectomized hosts even though the implants were allowed to remain only for a period of 10–14 days. Similarly, implantation of thymus tissue irradiated *in vitro* with either 500 or 2000 R restored the levels to normal in most of the recipients. The lymphocyte levels in the peripheral blood of mice in the various groups are shown in Fig. 1.

Antibody-plaque-forming cells

The experimental design for testing the capacity of thymectomized and thymus-grafted mice to produce antibody-plaque-forming cells following injection of sheep erythrocytes is shown in Table 1 and the results are presented graphically in Figs. 2 and 3.

Control mice not injected with sheep red cells were found in this and other experiments to produce less than one plaque-forming cell per million spleen cells. Sham-thymectomized mice injected with sheep erythrocytes produced an average peak titre of 220±25 (SE) plaque-forming cells 4 days after immunization at 28–31 days of age. Thymectomy at birth dramatically reduced this peak to 9±4·8 plaque-forming cells per million spleen cells. The difference between the peak titres of thymectomized and sham-thymectomized mice was highly statistically significant ($P<0·001$).

The presence for 10 days of an unirradiated thymus graft from either the parental strain, T6, or an unrelated strain, C3H, restored the capacity of thymectomized mice to produce a peak titre at 4 days of 72±18 and 47±11 plaque-forming cells per million spleen cells, respectively (Fig. 2). The difference between these peaks and the peak titre of 9±4·8 recorded in thymectomized controls is statistically significant ($P<0·01$).

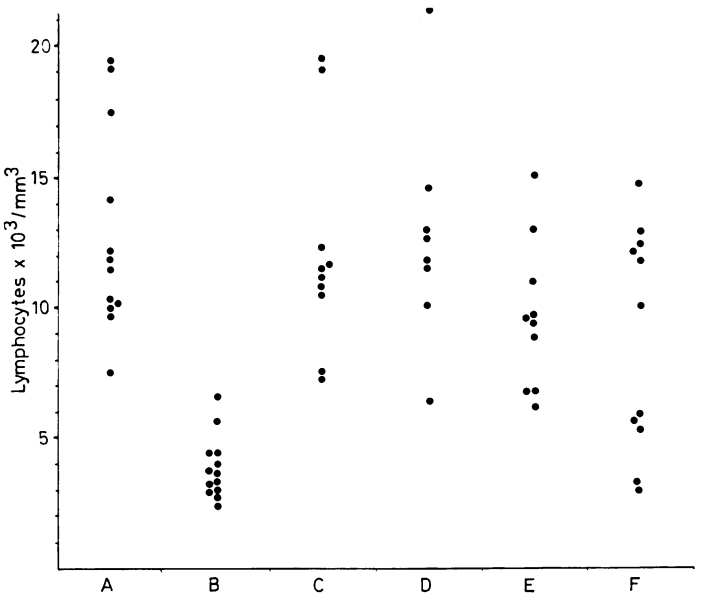


FIG. 1. Number of lymphocytes in the peripheral blood of groups of 2–4-month-old mice: A, sham-thymectomy; B, thymectomy only; C, thymectomy + T6 thymus graft in for 10–14 days; D, thymectomy + C3H thymus graft in for 10–14 days; E, thymectomy + T6 thymus irradiated with 500 R in for 10–14 days; F, thymectomy + T6 thymus irradiated with 2000 R in for 10–14 days.

TABLE 1. Experimental design for the detection of antibody-plaque-forming cells in thymectomized and thymus-grafted (Ak × T6)F_1 mice

Age (days)	Experimental					Controls	
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Birth	Thymectomy—all experimental mice (groups 1–5)					Sham-thymectomy (groups 6 and 7)	
7–10	Sham graft	Normal T6 thymus graft	Normal C3H thymus graft	500 R T6 thymus graft	2000 R T6 thymus graft	Sham graft	Sham graft
17–20	—	Thymus grafts excised (mice of groups 2–5)				—	—
28–31	Intraperitoneal injection of sheep erythrocytes (mice of groups 1–5)					I.P.I. sheep erythrocytes	I.P.I. saline
30–45	Spleen cells from mice in each group are plated out at intervals for estimation of number of antibody-plaque-forming cells						

The presence for 10 days of a T6 thymus graft, irradiated *in vitro* with 500 R, also restored to some extent the capacity of thymectomized mice to form plaque-forming cells: the peak titre in this group of mice was 36 ± 8 and the difference between this and the peak titre of thymectomized controls is statistically significant ($P < 0{\cdot}01$). By contrast, the presence for 10 days of a T6 thymus graft, irradiated *in vitro* with 2000 R, failed to restore immune capacity in thymectomized recipients (Fig. 3).

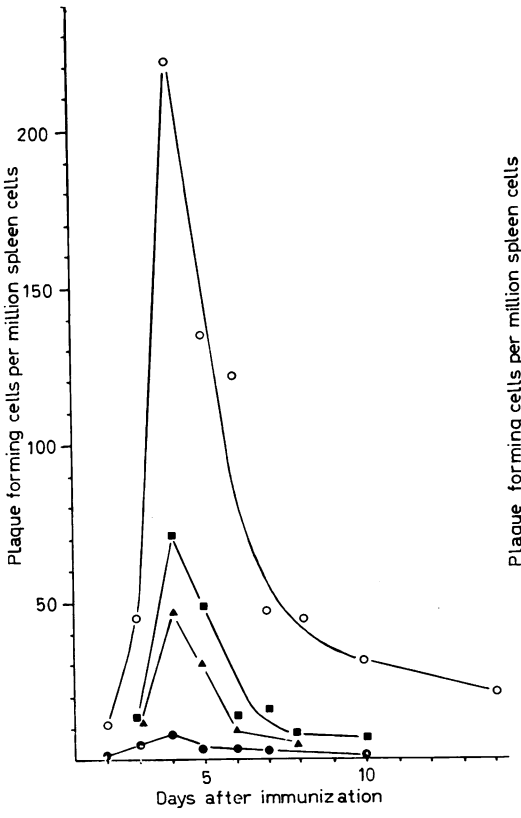


FIG. 2

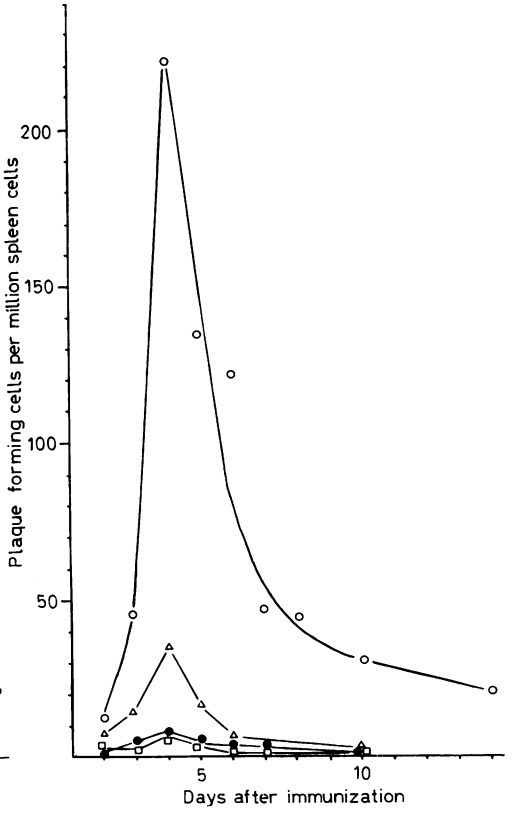


FIG. 3

FIGS. 2 and 3. Effects of thymectomy and unirradiated thymus grafts left in for 10 days on antibody-plaque-forming cells in spleens of groups of (Ak × T6)F_1 mice immunized at 28–31 days of age and killed between 30 and 45 days. Each point represents the average values obtained from four to twelve mice.

Fig. 2. ○, Sham-thymectomized at birth; ●, thymectomized at birth; ■, thymectomized at birth and grafted at 7–10 days with T6 thymus; ▲, thymectomized at birth and grafted at 7–10 days with C3H thymus.

Fig. 3. ○, Sham-thymectomized at birth; ●, thymectomized at birth; △, thymectomized at birth and grafted at 7–10 days with T6 thymus irradiated *in vitro* with 500 R; □, thymectomized at birth and grafted at 7–10 days with T6 thymus irradiated *in vitro* with 2000 R.

Skin graft survival

The effects of thymectomy and thymus grafting on the survival of C3H (H2k) and C57Bl (H2b) skin homografts on (Ak × T6)F_1 (H2k) mice are shown in Figs. 4 and 5. All the mice sham-thymectomized at birth rejected the foreign skin grafts within 20 days in marked contrast to mice subjected to total thymectomy at birth. The latter failed to reject the grafts which grew luxuriant tufts of hair and were present for about 2 months or more. All thymectomized mice bearing normal T6 thymus grafts for periods of 1, 2 or 3 weeks were capable of rejecting C3H and C57Bl skin grafts. The survival of these grafts was, however, prolonged in the mice in which thymus grafts were excised after 1 week. Full restoration

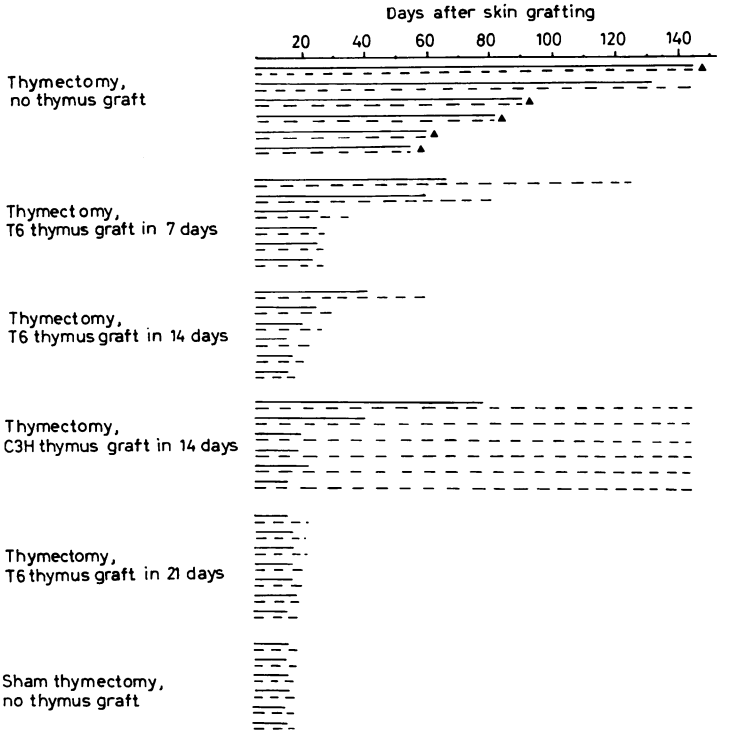


FIG. 4. Effects of thymectomy and implantation of unirradiated T6 and C3H thymus grafts on skin homograft rejection capacity in (Ak × T6)F_1 mice. ▲, Death from wasting disease. ——, C57Bl skin; – – –, C3H skin.

of homograft immunity occurred in most mice bearing thymus implants for 2 weeks and in all mice bearing implants for 3 weeks. The presence of a C3H thymus graft for 2 weeks enabled neonatally thymectomized (Ak × T6)F_1 mice to reject C57Bl skin but not C3H skin which was still present after an observation period of 140 days.

Most thymectomized mice bearing for 1 or 2 weeks thymus tissue, irradiated *in vitro* with 2000 R, failed to reject C57Bl and C3H skin grafts. Some, in which the implant was left in for a period of 3 weeks, rejected the grafts after a considerable interval of time (22–98 days). It seems therefore that irradiation of thymus tissue with 2000 R considerably impaired its capacity to restore homograft immunity. On the other hand, irradiation with

500 R did not significantly impair this capacity: mice bearing such thymus grafts for 2 weeks rejected C3H and C57Bl skin grafts within 20–60 days and those bearing the implants for 3 weeks rejected the skin within 14–25 days.

Wasting disease

As can be seen from Fig. 4, five of the six mice thymectomized at birth but not grafted with thymus tissue died of wasting disease between 80 and 175 days. Implantation of normal

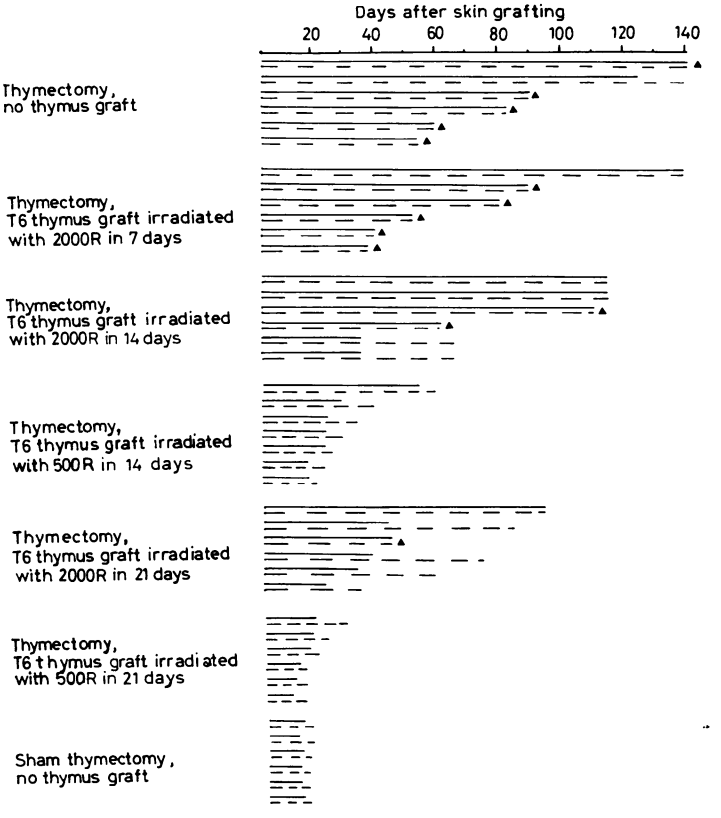


FIG. 5. Effects of thymectomy and implantation of T6 thymus grafts irradiated *in vitro* with 500 and 2000 R on skin homograft rejection capacity in (Ak × T6)F_1 mice. ▲, Death from wasting disease. ——, C57Bl skin; – – –, C3H skin.

thymus tissue completely prevented the development of wasting disease in the twenty-five mice used for skin grafting studies. This was the case whether the thymus was left in for periods of 1, 2 or 3 weeks and whether the thymus donor was the parental T6 strain or a foreign C3H strain. By contrast, wasting disease developed in five of six thymectomized mice bearing for 1 week thymus tissue irradiated *in vitro* with 2000 R, in two of six mice bearing the tissue for 2 weeks and in one of six mice bearing it for 3 weeks (Fig. 5). Implantation of thymus tissue irradiated with 500 R effectively prevented the development of wasting disease in the recipient mice.

Cytological analysis of the lymphoid tissues

The results of a cytological analysis of the lymph nodes and spleen of (Ak × T6)F_1 mice thymectomized at birth and grafted at 1 week of age with normal or irradiated (500 R) parental strain thymus tissue are shown in Table 2. The grafts were excised 7–10 days after implantation and the mice that had received normal and irradiated thymus tissue were further subdivided into four groups. One group (group 1) received no further treatment and was killed for cytological examination of lymph nodes and spleen at 7–9 weeks of age. Mice in group 2 were grafted with foreign skin (C57Bl) at about 5 weeks of age and killed at the height of the skin graft rejection for examination of the regional lymph node. Mice

TABLE 2. Cytological analysis of lymphoid tissues of 7–9-weeks-old neonatally thymectomized (Ak × T6)F_1 mice grafted at 1 week of age with newborn T6 thymus under the renal capsule

Treatment	Percentage of donor-type (thymus-derived) cells in:				
	Lymph nodes			Spleen	
	Unstimulated node (Group 1)	Regional node at height of skin graft rejection (Group 2)	Node draining area of trauma (Group 3)	Unstimulated (Group 1)	4 days after intraperitoneal injection of sheep erythrocytes (Group 4)
Thymectomy at birth; normal T6 thymus graft at 1 week; excision of graft 7–10 days later	3·2%*‡ (410/8)¶	20·7%*† (491/16)	8·3%†‡ (253/6)	0·4%§ (515/12)	2·3%§ (300/6)
Thymectomy at birth; 500 R irradiated T6 thymus graft at 1 week; excision of graft 7–10 days later	0% (289/6)	0% (323/6)	0% (301/6)	0% (200/6)	0% (255/6)

*$P<0·001$, †$P<0·001$, ‡$P<0·01$, §$P<0·02$ (χ^2-test).

¶Numerator = number of metaphases scored; Denominator = number of mice examined.

in group 3 had a piece of skin excised but no skin graft and were killed 7 days later for examination of the regional lymph node. Mice in group 4 received an intraperitoneal injection of sheep erythrocytes 4 days before killing and their spleens were analysed cytologically.

The great majority of the dividing cells in the lymph nodes and spleen of mice in group 1 which received a normal T6 thymus graft and no further treatment were of host-type: only thirteen thymus-derived cells were seen among 410 metaphases scored in the lymph nodes and two such cells among 515 metaphases scored in the spleen. Not one thymus-derived cell was found in the lymph nodes or spleen of the corresponding group of mice receiving irradiated thymus grafts.

At the height of skin graft rejection in the mice receiving a normal thymus graft the percentage of thymus-derived cells in the regional lymph node was 20·7%. Mice subjected to trauma *per se* had only 8·3% thymus-derived cells in their regional nodes. A small increase in the percentage of thymus-derived cells found in the spleen following injection of sheep erythrocytes ($P<0·02$).

No thymus-derived cell was ever found in the lymph nodes of mice bearing irradiated thymus grafts and either subjected to trauma or rejecting skin homografts nor in the spleens of those injected 4 days previously with sheep erythrocytes.

Cytological analysis of T6 thymus tissue irradiated with 500 R and implanted into neonatally thymectomized (Ak × T6)F_1 mice failed to reveal any donor-type dividing cells when examined from 2 to 21 days after implantation (Table 3).

TABLE 3. Cytological analysis of T6 thymus tissue irradiated with 500 R *in vitro* and implanted under the renal capsule of (Ak × T6)F_1 mice

Days after grafting	No. of grafts examined	Metaphases with donor cell characteristics (%)
2	2	—
4	2	—
6	3	—
8	4	—
10	4	—
12	3	0, 0, 0
14	4	0, 0, 0, 0
16	3	0, 0, 0

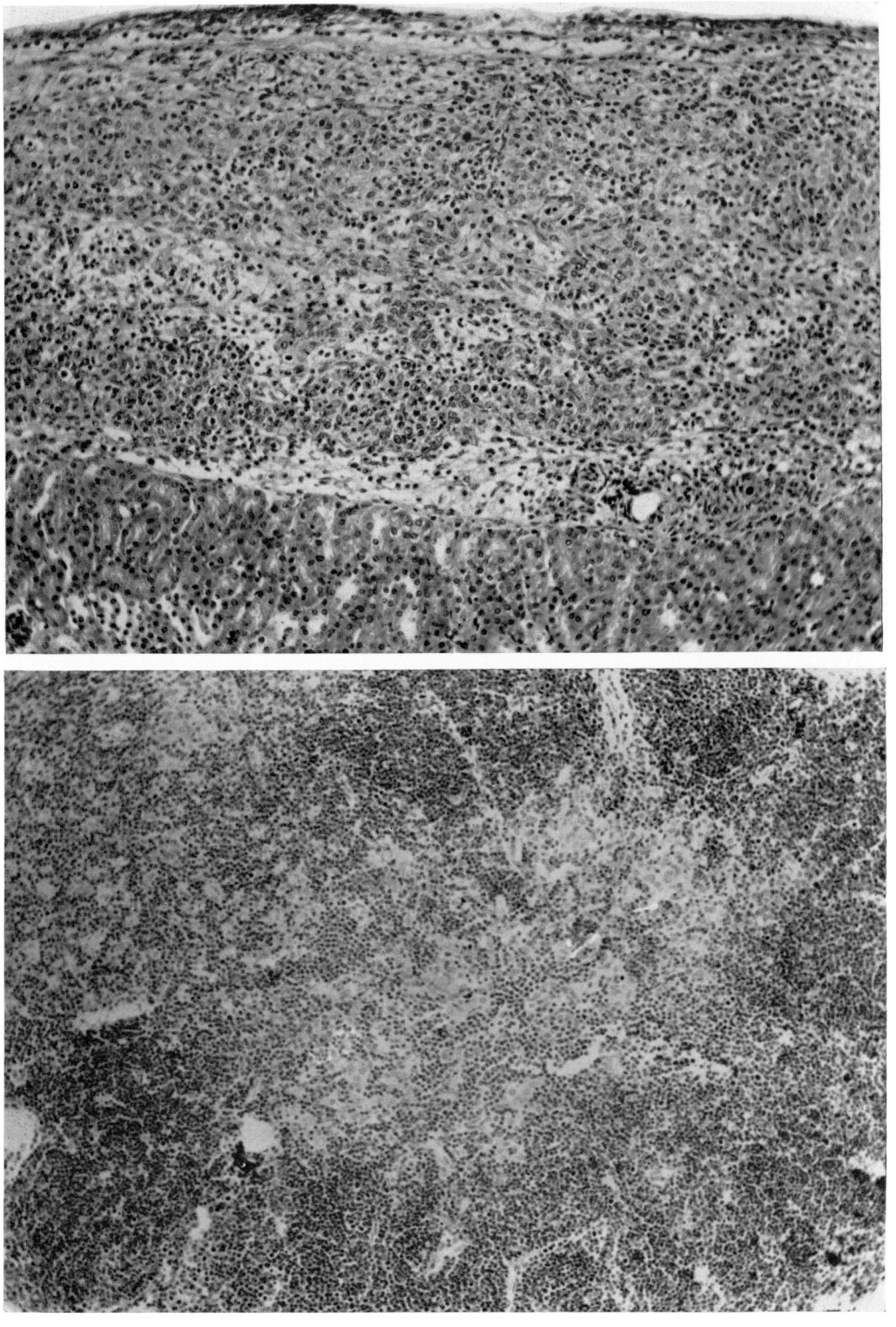
— = No scorable dividing cells in preparation.

Histology of thymus implants

Thymus implants excised from mice in the present experiments showed the same histological characteristics that were found in a previous study (Dukor *et al.*, 1965). Thymus tissue that had been irradiated with 500 R showed essentially the same regeneration pattern as was found in thymus grafts irradiated with 2000 R: for the first 4 days the implants were composed of epithelial-reticular framework totally devoid of lymphocytes; 'small lymphocyte-like cells' with dense chromatin structure appeared between 4 and 8 days; large

FIG. 6. Irradiated thymus tissue from newborn T6 donor 10 days after implantation under the renal capsule of neonatally thymectomized (Ak × T6)F_1 mouse. The graft consists of an epithelial-reticular framework in which are scattered 'small lymphocyte-like cells'. There are no typical thymus lymphocytes and little or no mitotic activity. Haematoxylin and eosin. ×155.

FIG. 7. Normal thymus tissue from newborn T6 donor 30 days after implantation under the renal capsule of a 7-day-old neonatally thymectomized (Ak × T6)F_1 mouse. The mice in this group were immunized with sheep red cells 4 days prior to sacrifice. Note normal thymus architecture and absence of germinal centres in the thymus graft. No plasma cells were seen. Methyl green pyronin. ×155.



FIGS. 6 (above) and 7 (below).

lymphoblast-like cells were first seen at 7–8 days but mitotic activity and medium and small lymphocytes were not evident until after 10 days (Fig. 6).

It was of interest to determine whether thymus grafts in immunized mice would show any of the histological changes characteristically seen in lymph nodes and spleen during the immune response. For this purpose twelve neonatally thymectomized $(Ak \times T6)F_1$ mice bearing normal T6 grafts in a subcutaneous site and under the renal capsule were immunized at 5 weeks of age with sheep erythrocytes and the grafts were removed for histological examination 4 days later. Germinal centres and plasma cells were not found in any of these grafts (Fig. 7).

DISCUSSION

The results presented here confirm those obtained in previous experiments showing: (1) that neonatal thymectomy greatly impairs homograft rejection capacity (not only between hosts and donors which share the same H-2 locus but also between those which differ at this locus (Miller, 1962a, b)), and practically inhibits the capacity to form antibody-plaque-forming cells to sheep erythrocytes (Takeya, Mori & Nomoto, 1964; Friedman, 1965; Miller, de Burgh & Grant, 1965); (2) that syngeneic thymus grafts enable neonatally thymectomized mice to develop capacities for certain types of immune reactions (Miller, 1962a, 1963); and (3) that allogeneic thymus grafts restore homograft immunity to third-party skin grafts but allow tolerance of skin grafts of thymus-donor-type (Miller, 1962b; Dalmasso *et al.*, 1963). In addition, the present study has established that normal (unirradiated) thymus grafts are capable of restoring immunological responsiveness in neonatally thymectomized mice within 7–14 days, and therefore before any significant number of host cells migrate into the graft, an event which, in subcapsular grafts, occurs at about 13 days after implantation (Dukor *et al.*, 1965). A similar conclusion has been reached in experiments in which thymus grafts were implanted into adult thymectomized irradiated hosts and excised after periods of 5, 10 and 15 days (Cross *et al.*, 1964).

Only very few thymus-derived cells were found dividing in the lymph nodes and spleen of unstimulated mice as reported previously (Dukor *et al.*, 1965). However, the percentage of those cells was increased in the lymph nodes draining a wounded area and considerably increased in those nodes regional to a skin homograft at the height of the rejection. A very slight increase in the percentage of thymus-derived cells also occurred in the spleens of mice injected with sheep erythrocytes 4 days previously. Direct evidence has thus been obtained for the migration of cells from a thymus graft present for only 7–10 days to sites of immunological activity. In parallel investigations on thymectomized irradiated adult mice bearing thymus implants it has also been found that the percentage of thymus-derived cells increased considerably in the spleen and lymph nodes following antigenic stimulation (Leuchars *et al.*, 1964, 1965).

Immunization did not, however, produce in either subcutaneous or subcapsular thymus grafts the histological changes characteristically seen in lymph nodes and spleen. No plasma cells nor germinal centres were ever seen in these implants which were present at the height of the immune reaction. This is in contrast to what has been reported by Stutman & Zingale (1964) who described germinal centre formation and a marked plasma cell proliferation in subcutaneous thymus autografts of young adult rats immunized with diphtheria toxoid.

Using a similar system neither Metcalf (1965, personal communication) nor our group (Miller & Grant, 1965) could repeat these observations.

The cytological results represented here might, at first sight, suggest that the restoration of immune capacities in neonatally thymectomized mice depends exclusively on cells produced by the thymus implant which migrate out and are capable of responding to an antigenic stimulus by proliferating in the lymph nodes and spleen. The significance of the increase in the percentage of thymus-derived cells in such areas after antigenic stimulation has not however been elucidated. It does not necessarily follow that these cells are directly (or indirectly) involved in antibody production or skin homograft rejection. In fact, experiments in which thymus-derived cells were inhibited in specifically isoimmunized hosts have shown that their presence is not essential for immunological activity (Leuchars *et al.*, 1965). It would seem therefore that the increase in the proportion of thymus-derived cells in the spleen and lymph nodes of antigenically stimulated animals reported in this study and in the studies on thymectomized irradiated adult mice (Leuchars *et al.*, 1964, 1965) is an incidental finding that has no strict relevance to the essential mechanism by which thymus implants reverse the immunological defects of thymectomized mice.

The presence for a period of 1, 2 or 3 weeks of thymus implants irradiated *in vitro* with 2000 R failed to reverse completely the immunological defects of neonatally thymectomized mice. Evidence of partial restoration was obtained mostly in the group of mice in which the implant resided for 3 weeks: wasting disease was considerably reduced and skin homografts were eventually rejected by some of these mice. Investigations on mice receiving a potentially lethal dose of total body irradiation (850 R) have likewise revealed that restoration of immunological responsiveness by the irradiated thymus *in situ* does not begin until some 2 weeks after irradiation (Cross *et al.*, 1964). In a previous study (Dukor *et al.*, 1965) it was shown that thymus implants, irradiated *in vitro* with 2000 R were totally devoid of lymphocytes for the first 4 days, that no dividing cell of donor-type could be identified at any time in the grafts, that host-type cells appeared in the graft in increasing numbers after 11 days and that typical thymus lymphocytes and arrangement into cortico-medullary areas did not take place until after 12–15 days. It could be argued, therefore, that the failure of heavily irradiated implants to effect any restoration of immunological capacity after a period of 2 weeks is a reflection of the absence of typical thymus lymphocytes in the grafts during that period. The implants can regain their complement of lymphocytes only after the immigration of host lymphoid precursor cells which differentiate within the implant and proliferate to form typical thymus lymphocytes. According to this view, restoration of immunological responsiveness could be effected only after thymus lymphocytes become available for distribution from the implant. Experiments with thymus implants irradiated *in vitro* with 500 R have however shown that restoration of immunological responsiveness has been initiated after a period of 10–14 days. An appreciable number of antibody-plaque-forming cells were present in the spleens of mice bearing such implants for 10 days and skin homografts survived only slightly longer than normal in most mice bearing the grafts for only 14 days. It could be argued that some thymus lymphocytes escaped destruction after 500 R whereas none were spared after 2000 R and, therefore, that the earlier and more effective restoration of immune capacities by thymus implants exposed to 500 R depended on the presence of such lymphocytes which became readily available for distribution to the host. Against this hypothesis are the following observations: (1) It has been shown that

thymus lymphocytes are virtually destroyed by exposure to 500 R (Trowell, 1961); (2) No donor-type dividing cells were ever found in the implants irradiated with 500 R, nor in the spleens and lymph nodes of stimulated mice; and (3) no typical thymus lymphocytes could be seen during the first week after implantation of these thymuses. In fact, the regeneration pattern of implants irradiated with 500 R was essentially similar to that of implants irradiated with 2000 R. Furthermore, in both situations the grafts were completely regenerated by 3 weeks and filled with actively dividing host-derived cells and yet practically full restoration of the capacity for homograft immunity was evident only in mice bearing thymus tissue irradiated with 500 R; those bearing heavily irradiated thymus tissue were still considerably deficient in their capacity to reject homografts.

The failure of thymus implants irradiated with 2000 R to restore immunological responsiveness significantly may thus be related not to the delay in appearance of thymus lymphocytes but rather to an impairment of the capacity to induce differentiation or maturation of immunologically competent cells from noncompetent precursors. This property of thymus tissue may be dependent on the integrity of the thymus epithelial-reticular cell complex. It has been reported that thymus epithelial-reticular cells are completely resistant to doses of X-irradiation up to 5000 R (Trowell, Corp & Lush, 1957; Trowell, 1961) but the results presented here show that this is not entirely true. Irradiation with 2000 R has impaired the ability of the thymus to reverse the immunological defects of neonatally thymectomized mice. It is possible that these cells elaborate a humoral factor which is essential for the maturation of lymphoid cells to immunologically competent cells (Osoba & Miller, 1963, 1964; Miller, 1964). This function of the epithelial-reticular cells may not be resistant to doses of radiation in the vicinity of 2000 R and above.

There is a hint in the present work of a dissociation between thymus-dependent lymphopoiesis and thymus-dependent immunological capacity. As mentioned above, lymphopoiesis was active in thymus implants which had received 500 R as well as in those which received 2000 R; yet only the former significantly restored immunological responsiveness. Furthermore, blood lymphocyte levels were within the normal range in most mice thymectomized at birth and bearing either normal thymus implants or implants irradiated *in vitro* with 500 and 2000 R. These observations clearly indicate that blood lymphocyte levels do not necessarily reflect the state of immunological capacity. A similar conclusion was reached in studies of the effect of thymus tissue enclosed in millipore diffusion chambers (Osoba & Miller, 1963, 1964; Osoba, 1965). Of the mice restored in their capacity to produce immune responses, only a small number had a normal population of lymphocytes; the majority still showed a moderate or marked depletion of lymphocytes in both blood and lymphoid organs.

The essential functions of the thymus may thus be (1) to provide lymphocytes by trapping lymphoid precursor cells and causing, within the thymus environment, their differentiation and proliferation to noncompetent lymphocytes, and (2) to elaborate a factor (a competence-inducing-factor (Miller, 1965b)) which directs the maturation (or differentiation) of noncompetent cells (presumably lymphocytes) to immunologically competent cells. The results presented above, when taken together with the work on diffusion chambers, suggest that this competence-inducing function of the thymus is dependent on the integrity of the epithelial-reticular cells but that the acquisition of full immunological competence is an event which does not necessarily take place only within the environment of the thymus.

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