TRANSLANTATION OF CULTURED THYMIC FRAGMENTS

II. Results in Nude Mice*

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Attempts to correct the deficient T-lymphoid system of nude mice have utilized intact thymuses, nonlymphoid epithelial thymus grafts, as well as suspensions of intact cells (1–9). Variable degrees of success have been reported but generally, the responses of the transplanted animals are inferior to normals of the same strain (6, 7). To date the best results have been reported with intact thymuses obtained from syngeneic neonatal donors (7). In attempts to correct human combined (both B and T) immunodeficiency disorders, fetal thymus transplants have been employed in cases where the ideal reconstituting modality, histocompatible bone marrow, has not been available (10–12). Only modest changes in some of the in vitro tests of T-lymphocyte function have resulted. More recently, we have employed transplants of cultured thymic fragments in cases of combined immunodeficiency where no bone marrow donor was available (13, 14). The initial results have been quite encouraging (14) and laboratory studies are presently underway to attempt to define the optimal conditions for culturing the glands and transplanting the tissue.

In our initial studies, we utilized normal mice to show that animals with normal prethymic stem cell compartments could repopulate transplanted thymic fragments which had been depleted of lymphocytes and macrophages by a short period of organ culture (15). These studies indicated that indeed the cultured thymic fragments could become lymphoid and were morphologically normal. In the series of experiments to be described here, we have studied the ability of cultured thymic fragments to reconstitute nude mice. Both allogeneic and syngeneic transplants have been performed and significant reconstitution of the nudes has been achieved.

Materials and Methods

Mice. Nude mice were obtained by breeding heterozygous (nu/nu + ) females to homozy- gous (nu/nu) males, all of which were on the BALB/c/BOM (Ry, Denmark) background. HaICr and normal BALB/c newborn mice were a generous gift of Dr. R. Auerbach, University of Wisconsin.

Thymus Culture. Thymuses were removed from syngeneic (BALB/c, KI, DBA/2; H-2d) or allogeneic (HaICr, H-2q) newborn mice immediately after cervical dislocation of the donors. The thymuses were cut into small fragments and placed on metal organ culture grids (organ culture grids, 60 mesh, BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). Each thymus lobe was cut into two to three pieces. No attempt was made to strip the capsule. Organ cultures were initiated in Ham's F-12 medium containing 10% fetal calf serum. For the first 24-h, the fluid level was just touching the fragments. Thereafter, when the

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pieces were adherent to the grids, culture medium was added to cover all pieces by 2-3 mm. Tissues were cultured in a humid environment at 37°C in 95% air and 5% CO2; medium was changed at 72 h and at 1 wk. Samples were taken at frequent intervals for frozen section and when thymocyte and macrophage depletion was judged to be as complete as possible without notable deterioration of epithelial components, the tissues were transplanted. Previous experiments, employing transplantation into normal animals as well as chemotaxis experiments have indicated that too lengthy a culture period results in decreased chemotactic capability of the fragments and poor lymphoid repopulation in vivo (15). The usual time of culture was 7-8 d.

**Transplantation.** When the cultured fragments were judged to be ready for transplant (Figs. 1-3), usually when the lymphocytes were virtually all gone or appeared quite pyknotic (7-8 d), the recipients were anesthetized with chloral hydrate, and transplantation under the renal capsule performed. Approximately 12-18 fragments were transplanted on each side, representing the tissue originally obtained from three donors.

**H-2 Typing.** H-2 allotyping sera were obtained from the National Institutes of Health. Cells were typed by means of a cytotoxicity assay, using trypan blue exclusion as a sign of viability (16). Cytotoxicity due to rabbit complement alone, or antisera without complement was always less than 5%. The specificity of the antisera was shown to be at least 95% or greater.

**Lymphocyte Stimulation.** Proliferative assays employed spleen cells or lymph node cells from tissues removed at autopsy. The lymphoid cells were teased from the tissues and incubated in RPMI-1640 with 3% inactivated fetal calf serum at 37°C in an atmosphere of 5% CO2 for 3 d. [3H]thymidine was added 18 h before harvest, 1 μCi per test well. Tests were performed in triplicate and utilized 5 × 10⁶ mononuclear cells. Phytohemagglutinin (Difco Laboratories, Detroit, Mich.) was employed in doses of 0.6, 0.8, 1.0, and 1.2 μg, concanavalin A (Calbiochem, San Diego, Calif.) 0.5, 1.0, 1.5, and 2.0 μg, and lipopolysaccharide (Sigma Chemical Co., St. Louis, Mo.) at 0.6, 0.8, 1.0, and 1.2 μg. Mixed leukocyte culture was performed by using 5 × 10⁶ stimulator and 5 × 10⁶ responder cells; stimulators were radiated with a cesium source for a total of 2,000 rads (17). Cell-mediated lympholysis was performed as described by Zarling et al. (18).

**Skin Grafting.** Skin grafts were performed according to the cyanoacrylate cementing technique of Rygaard (19) as modified by Manning and Krueger (20). Grafts were examined daily from day 6 onward to insure that vascularization had occurred. In no case was vascular compromise noted indicating lack of technical failures. Rejection was judged visually and defined as separation of a totally indurated graft from its bed.

**Histology.** Tissues were embedded in paraffin and routine 6 μm hematoxylin and eosin sections were examined. In some cases, tissues were embedded in J-B4 embedding medium, cut at 1-2 μm, and stained with hematoxylin and eosin. At approximately monthly intervals, animals were bled from the retroorbital sinus. Differential counts, total leukocyte counts, surface immunoglobulin bearing (SIg+) cells were determined. SIg+ cells were demonstrated by the method of Pepys et al. (21) using reagents prepared by ourselves. Thy-1 cells were detected with rabbit antiserum purchased from Cappel Laboratories, Inc., Cochranville, Pa. using a cytotoxicity assay (16).

**Serum Immunoglobulins.** IgG, IgM, and IgA were estimated by noting the highest dilution of serum which resulted in a precipitin arc as seen in gel diffusion against monospecific anti-heavy-chain antisera (Meloy Laboratories, Inc., Springfield, Va.), according to the technique of Arnason et al. (22).

**Antibody Titers.** Sheep erythrocyte titers were measured in serum at 5 d after primary and secondary injections of 10⁸ sheep erythrocytes intraperitoneally. Titters were performed in microtiter plates as described by Wegmann and Smithies (23). Dithiothreitol resistant and sensitive antibodies were determined by performing the assays in the presence and absence of 0.2 M dithiothreitol. Resistant antibodies were assumed to be of the IgG class. Precipitating

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1 H. Schulte-Wissermann. et al. Functional relationship of macrophages and basophils to the thymus gland. *Scand. J. Immunol.* In press.

2 We are indebted to Ms. Carol Jordan, Mr. AI Kutchera, and Dr. Ralph Albrecht for help in thin sectioning of tissues.

3 Abbreviations used in this paper: ANA, anti-nuclear antibodies; CML, cell-mediated lympholysis; CTF, cultured thymic fragments, SIg+, surface immunoglobulin bearing.
antibodies to rabbit serum proteins were measured after 4 i.p. injections of 0.2 ml of normal rabbit serum. Titers were determined by using progressive dilutions of the test serum, and noting the highest dilution which resulted in a visible arc(s) in gel diffusion against normal rabbit serum diluted 1:4 as the antigen.

Anti-Nuclear Antibodies (ANA). ANA were sought for using frozen sections of rat stomach as a source of nuclei, mouse serum samples from the experimental groups diluted 1:10, followed by goat anti-mouse gamma globulin, fluorescein conjugated at a molar ratio of 2:1. Normal mouse serum similarly diluted, was used as a control and was always negative. Fluorescent brightness was grossly estimated from 0 to 3+

Results

During the period of culture, the macrophages and lymphocytes leave the culture or die in situ (15). As shown in Figs. 1–3, the fragments become progressively depleted. This process is largely complete by 7–8 d of culture; a few thymocytes remain, but they are quite pyknotic. When fragments are teased apart at this time, the lymphocytes are virtually all dead as indicated by lack of vital dye exclusion.

A variable sized lymphoid transplant was found in all transplanted animals except one. Although the grafts were larger than at the time of transplant, from 8 to 10 mo before, the aggregate mass was not nearly that of the thymus of a normal animal.

All grafts were abundantly lymphoid and corticomedullary differentiation was evident (Figs. 4–6).

No normal thymic tissue was observed in the mediastinum of any nude mouse at the time of autopsy. Paired bodies (9) were found in three of four nontransplanted nude mice. They were always quite small and found only on careful dissection of the mediastinum. Histologically, they were alymphoid and similar to those previously described by Wortis et al. (9). In five of nine transplanted animals, a very large cystic structure, occupying the anterior mediastinum and overlying the heart in the usual position of a normal thymus was seen. Histologic examination showed essentially the
FIG. 2. Mouse thymus fragment, day 4 of culture. Depletion of lymphocytes has begun. × 100.

FIG. 3. Mouse thymus fragment, day 7 of culture. Only a small number of lymphocytes are present. Most cells are epithelial. × 100. Lymphocytes teased from such a fragment at this time will not exclude trypan blue.

same morphology as the usual paired bodies, but the cysts were larger (Fig. 7). There was no lymphocyte infiltration whatever.

All transplanted animals showed progressive weight gain (7–12 g, mean = 8.2 g) and were spared of wasting. Their health remained good until the time of sacrifice.
FIG. 4. Subrenal capsule transplant of allogeneic cultured thymic fragment into nude mouse 10 mo after implantation. Corticomedullary differentiation is apparent. × 100.

FIG. 5. High-power view of transplant shown in Fig. 4, showing epithelial cells in medulla. × 400.
Fig. 6. High-power view of transplant shown in Fig. 4, showing thymocytes in cortex. × 400.

Fig. 7. Paired body obtained from a nude mouse transplanted with syngeneic cultured thymus. No lymphocytic infiltration seen. The organ was enlarged due to extensive cyst formation. × 100.
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**Table I**

*Leukocyte Counts in Peripheral Blood*

| Thymus source                  | Time after transplant | Total leukocyte (*/mm³*) | Time after transplant | Total lymphocyte (*/mm³*) |
|--------------------------------|-----------------------|-------------------------|-----------------------|-------------------------|
|                                | 0                     | 3 mo                    | 8 mo                  | 0                      | 3 mo                    | 8 mo                  |
| Syngeneic cultured thymus      | 2,700                 | 3,000                   | 3,900                 | 2,130                  | 2,670                   | 3,590                 |
|                                | 3,200                 | 2,600                   | 3,500                 | 2,980                  | 2,440                   | 3,330                 |
|                                | 3,300                 | 2,800                   | 3,900                 | 2,510                  | 2,490                   | 3,820                 |
| Allogeneic cultured thymus     | 3,700                 | 2,800                   | 3,800                 | 1,670                  | 2,160                   | 3,270                 |
|                                | 2,800                 | 2,400                   | 4,600                 | 2,040                  | 2,040                   | 4,000                 |
|                                | 3,700                 | 2,600                   | 4,000                 | 3,030                  | 1,850                   | 3,720                 |
|                                | 2,700                 | 2,200                   | 4,500                 | 1,070                  | 1,780                   | 4,010                 |
|                                | 2,100                 | 2,000                   | 4,100                 | 1,370                  | 1,620                   | 3,900                 |
|                                | 1,800                 | 2,600                   | 4,400                 | 1,100                  | 2,180                   | 4,220                 |

* Normal BALB/c (mean of five animals): total leukocytes = 8,860/mm³; total lymphocyte count = 7,903/mm³.

**Table II**

*Surface Markers in Peripheral Blood*

| Thymus source                  | IgM (*/mm³)* | Time after transplant | IgG (*/mm³)* | Time after transplant | Thy-1 (*/mm³)* | Time after transplant |
|--------------------------------|--------------|-----------------------|--------------|-----------------------|---------------|-----------------------|
|                                | 0            | 3 mo                  | 8 mo         | 0                     | 3 mo          | 8 mo                  |
| Syngeneic cultured thymus      | 190          | 130                   | 490          | 560                   | 680           | 560                   | 950                   |
|                                | 190          | 210                   | 320          | 250                   | 1,440         | ND                    | ND                    | 940                   |
|                                | 170          | 80                    | 430          | 260                   | 620           | ND                    | ND                    | 740                   |
| Allogeneic cultured thymus     | 190          | 110                   | 490          | 560                   | 680           | 60                    | 560                   | 950                   |
|                                | 220          | 100                   | 280          | 530                   | 920           | 0                     | 550                   | 1,380                 |
|                                | 70           | 100                   | 440          | 140                   | 310           | 600                   | 30                    | 1,200                 |
|                                | 220          | 110                   | 360          | 320                   | 350           | 540                   | ND                    | 770                   |
|                                | 20           | 100                   | 620          | 230                   | 280           | 900                   | ND                    | 980                   |
|                                | 20           | 130                   | 440          | 90                    | 260           | 700                   | ND                    | 1,060                 |

* Normal BALB/c (mean of five animals): IgM = 738/mm³; IgG = 1,359/mm³; Thy-1 = 2,115/mm³.

ND, not done.

All nontransplanted animals died by 4 mo of age. The animals were not kept in a germ-free environment.

Lymphocyte counts increased progressively in all transplanted animals, reaching maximal levels approximately 8 mo after transplantation (Table I). Thy-1 bearing cells were observed in gradually increasing numbers in the peripheral blood beginning about 3–4 mo after transplant (Table II). Total lymphocyte counts for all transplanted animals, whether using noncultured, cultured syngeneic or cultured allogeneic grafts are less than normal values, however (Tables I and II).

Lymphoid tissues of the transplanted animals were normal at the time of autopsy. The T-dependent areas, periarteriolar sheaths of the spleen and paracortical zones of lymph nodes, were rich in lymphocytes. Lymph nodes showed well developed secondary follicles. Untransplanted nude mice showed deficient T-dependent lymphocyte zones (Figs. 8–11).

Allogeneic second party skin grafts (of the thymus donor strain) were applied in six cases and syngeneic grafts in three. Allogeneic second party grafts were not acutely
Fig. 8. Periarteriolar sheath showing abundant T lymphocytes in spleen of nude mouse transplanted with allogeneic cultured thymus fragment. × 100.

Fig. 9. Periarteriolar sheath of untransplanted nude mouse. Arteriole shown by arrow. Lack of T-dependent lymphocytes is apparent. × 100.
rejected, but luxuriant hair growth seen with syngeneic grafts was not observed. An allogeneic third party (i.e. different from recipient and donor of thymus) graft was applied approximately 3-4 wk later and was rejected in all (nine of nine) cases (Table III).

In vitro tests of lymphocytes were performed on spleen and lymph nodes as terminal experiments. The age of the animals varied from 10 to 11 mo and the time from the date of transplant from eight to ten mo. The results are shown in Table IV. In general, lymph node lymphocytes are more responsive than splenic lymphocytes both in normal and transplanted nudes. Nontransplanted nude mice showed virtually no response. It can be seen that proliferative responses to phytohemagglutinin tend to be less vigorous in the transplant recipients than in the normal. There is no difference
between animals reconstituted with syngeneic or allogeneic tissues, however. Responses to concanavalin A were as good as or better than the normal control in three of nine and somewhat less but definitely positive in the rest. On the whole, lipopolysaccharide responses were greater than in the normal, perhaps reflecting a higher B-cell content of nude lymphoid tissues or a deficient regulatory T-cell population. Allogeneic cell responses were usually vigorous and better than or equivalent to the normal control in four of the nine. Only two of nine showed poor responses. Three animals were nonresponsive in cell-mediated cytology assessments, although they showed brisk stimulation by the target cells. One animal showed a weak proliferative allogeneic cell response (SI = 4.0) but an excellent CML reaction (21% killing). In general, spleen cells showed similar trends, although the degree of response tended to be of much lower magnitude. When compared with lymphocyte responses of nude mice transplanted with newborn noncultured whole thymuses, animals reconstituted with cultured fragments show more vigorous responses.

B-cell responses are shown in Table V. IgG1 and IgA levels are most profoundly affected in untreated nude mice (24) and were brought to near normal or normal levels in all transplanted animals. Significant levels of antibodies to rabbit serum proteins was observed as well as dithiothreitol resistant (IgG) antibodies to sheep erythrocytes. Both of these responses are T dependent (8); D. D. Manning, unpublished observation.

| Host          | Thymus donor | Skin donor | Graft survival |
|---------------|--------------|------------|----------------|
| BALB/c (H-2d)| DBA/2 (H-2d) | BALB/c     | >8 mo          |
|               | DBA/2        |            | >8 mo (?)*     |
|               | AKR (H-2k)   |            | <12 d          |
| BALB/c (H-2d)| KI (H-2d)    | BALB/c     | >8 mo          |
|               | KI           |            | >8 mo (?)*     |
|               | AKR          |            | <12 d          |
| BALB/c (H-2d)| HAICr (H-2q)| HAICr      | >8 mo (?)*     |
|               |              | C57 Bl 6 (H-2b) | <12 d        |
| BALB/c (H-2d)| HAICr        | HAICr      | >8 mo (?)*     |
|               |              | C57 Bl 6   | <12 d          |
| BALB/c (H-2d)| HAICr        | HAICr      | >8 mo (?)*     |
|               |              | C57 Bl 6   | <12 d          |
| BALB/c (H-2d)| HAICr        | HAICr      | >8 mo (?)*     |
|               |              | C57 Bl 6   | <12 d          |
| BALB/c (H-2d)| HAICr        | HAICr      | >8 mo (?)*     |
|               |              | C57 Bl 6   | <12 d          |
| BALB/c (H-2d)| HAICr        | HAICr      | >8 mo (?)*     |
|               |              | C57 Bl 6   | <12 d          |

* See text for remarks concerning survival of graft.
**Table IV**

|                | PHA* | Con A* | LPS* | MLC* |
|----------------|------|--------|------|------|
|                | MMSI | cpm × 10⁻³ | MMSI | cpm × 10⁻³ | MMSI | cpm × 10⁻³ | SI | cpm × 10⁻³ | CML* |
| Exp. 1         |      |         |      |      |      |      |      |      |      |
| Normal (8)     | 54.1 ± 26.6 | 82.5 ± 38.9 | 12.4 ± 6.1 |      |
| Nontransplanted| 1.3 ± 0.89 | 1.3 ± 0.89 |      |      |
| Nudes (6)      | 1.4 ± 0.91 | 1.48 ± 0.84 | 2.9 ± 1.2 |      |
| Exp. 2         | 53   | 45.7/0.86 | 28.4 | 24.5/0.86 | 3.3  | 2.8/0.86 | 9.2 | 2.13/0.83 |
| Syngeneic whole thymus transplant** | 1.2 | 24.3/0.84 | 1.9 | 38.0/0.84 | 1.4  | 28.8/0.84 | <1 | 6.8/7.8   |
| Exp. 3         | 73.1 | 119.1/1.6 | 24.6 | 39.9/1.6 | 1.9  | 3.1/1.6 | 36.8 | 6.9/0.3   |
| Syngeneic CTF  | 17.6 | 106.1/1.1 | 31.6 | 33.3/1.1 | 3.2  | 3.5/1.1 | 27.2 | 3.0/0.11  |
| transplant† | 9.5  | 37.6/0.9 | 4.9  | 19.6/0.9 | 6.7  | 24.7/0.9 | 61.8 | 15.6/0.5  |
| Allogeneic CTF | 29.8 | 174.3/5.8 | 13.8 | 61.0/5.8 | 3.8  | 22.0/5.8 | 46.4 | 15.8/0.8  |
| transplant‡ | 2.3  | 29.2/1.8 | 1.9  | 34.7/1.8 | 1.6  | 23.7/1.8 | <1  | 7.7/10.1  |
| Exp. 4         | 19.8 | 123.8/6.2 | 22.5 | 141.0/6.2 | 2.1  | 13.2/6.2 | 77.8 | 28.6/0.3  |
| Allogeneic CTF | 3.6  | 49.5/13.0 | 5.1  | 70.3/13.0 | 1.7  | 28.6/13.0 | 3.8 | 9.3/2.4   |
| transplant§ | 4.6  | 50.5/11.0 | 5.4  | 59.8/11.0 | 2.1  | 23.2/11.0 | 4.0 | 17.8/4.4  |
| 26.3 | 14.1/6.0 | 17.7 | 55.7/3.1 | 2.3  | 7.7/3.1 | 19.0 | 7.8/0.4   |

* PHA, phytohemagglutinin; Con A, concanavalin A; LPS, lipopolysaccharide; MLC, mixed leukocyte culture.
† MMSI, maximal mitogenic stimulation index: the maximal stimulation ratio of experimental over nonstimulated tritium incorporation observed with variable mitogen doses used.
‡ cpm × 10⁻³, actual counts per minute from which MMSI or SI derived.
§ SI, stimulation index: observed counts produced by culture of stimulator (radiated) and responder cells divided by counts produced by a mixture of radiated and nonirradiated stimulator cells alone.
¶ Data in exp. 1 are shown as the average number of counts ± the standard deviation for all animals in the group. The number of animals in each study is shown in the parenthesis. MLC data for exp. 1 are shown as the average stimulation index of the group. In other experiments, individual results for each animal are shown.
** Nude mice transplanted with uncultured newborn syngeneic thymus implanted intraperitoneally.
†† Nude mice transplanted with syngeneic cultured thymus fragments.
‡‡ Nude mice transplanted with allogeneic cultured thymus fragments.

H-2 typing of the lymph node and spleen lymphocytes could be performed in five of six allogeneic transplant recipients. All of the lymphocytes were shown to be of host origin by this method and no evidence for persistent donor cells was found.

**Discussion**

The transplants prolonged the lives of all recipients. None of the untransplanted nude mice lived over 4 mo. Nine of nine cultured thymic fragment (CTF) transplanted nude mice were alive and apparently well from 10 to 11 mo after the transplant. Weight gain was progressive to about 30-35 g, after which a stable weight was maintained. There was no evidence of wasting.

Taken together, the results described above show that the cultured epithelial fragments retain the capacity to attract and differentiate stem cells of the nu/nu recipients to yield competent T cells. It is clear that the reconstitution is due to host cells because typing of lymphoid tissue containing immunologically reactive cells reveals cells to be 100% of host strain. These newly acquired T cells have demonstrable killer and helper capability. The evidence for the helper cell reconstitution is the
acquisition of normal serum levels of IgA and IgG1 and the development of functional antibodies (Anti-rabbit serum protein and IgG anti-sheep erythrocyte were found at control levels). These B-cell functions are known to be thymus dependent (8, 26). T-killer function could be shown in vitro by cell-mediated cytolysis and in vivo by vigorous skin allograft rejection. The development of functional capability is especially important in considering this treatment in the restoration of human deficiency disease. Except for bone marrow transplantation, transplants of other lymphoid tissues in man (e.g., fetal liver or fetal thymus) have usually improved functional capacity little or none.

Controversial results concerning the rejection of skin of the thymus donor have been reported in previous transplant studies, using allogeneic mouse thymus donors, (2, 25–28). Our studies are only preliminary on this point, and we believe that further more extensive testing will have to be done to answer this intriguing question. We cannot state that complete acceptance of thymus donor skin occurred since hair growth was not observed. However, hair growth can be impaired even when rejection does not occur, and is not, therefore an unequivocal sign of graft acceptance (19). Extensive testing with many well defined strains will be necessary. In one experiment, we found that the recipient was able to respond with proliferation after stimulation in vitro by cells of the thymus donor. The degree of proliferation (SI = 4.5 ×) was marginal; however, the mixed leukocyte culture harvesting was not performed until after 5 d of culture, perhaps too late for the peak response of a secondarily stimulated

### Table V

| Group | Test Group | IgG1 | IgG2 | IgA | IgM | Anti-rabbit* | Anti-SRBC§ |
|-------|------------|------|------|-----|-----|--------------|------------|
| Group 1 | nu/+ (7)§ | 622 | 15 | 0 | 0 | 0 | 0 |
|        | nu/+ (8)§ | 1084 | 316 | 11 | 86 | 30 | 30 |
|        | nu/nu (6)** | 8 | 250 | 4 | 80 | 0 | 0 |
| Group 2 | Syngeneic W. T. §§ (3) | 1600 | 464 | 33 | 80 | 19 | 19 |
| Group 3 | Syngeneic CTF*** (3) | 384 | 256 | 8 | 10 | 16.5 | 16.5 |
| Group 4 | Allogeneic CTF†† (6) | 736 | 384 | 42 | 29 | 20 | 20 |

* Rabbit serum injected i.p. and titers measured by Ouchterlony immunodiffusion. The number shown is the mean of the reciprocal titer at which the precipitin lines disappear. Numbers in parentheses are the range of values for each group.

§ Antibodies to sheep erythrocytes measured by hemagglutination in the presence or absence of dithiothreitol (DTT). Number shown is the mean of the reciprocal log2 titers; range of values is shown in parentheses.

§§ Heterozygote litter mates of (***) and (†††) not injected with rabbit serum. Number of animals in each experiment shown in parentheses.

† The mean of the reciprocal dilution titer at which the precipitin line was no longer seen. The range for all animals in the category is shown in parentheses.

‡ Heterozygote litter mates (***) and (†††) injected with normal rabbit serum.

§§§ Nude mice, nontransplanted but injected with rabbit serum.

∗∗∗ Nude mice, nontransplanted, not injected with rabbit serum.

†††† Nude mice transplanted with syngeneic whole noncultured thymic fragments.

### Additional Notes

- Acquisition of normal serum levels of IgA and IgG1 and the development of functional antibodies (Anti-rabbit serum protein and IgG anti-sheep erythrocyte were found at control levels).
- B-cell functions are known to be thymus dependent.
- T-killer function could be shown in vitro by cell-mediated cytolysis and in vivo by vigorous skin allograft rejection.
- Functional capability is especially important in considering this treatment for human deficiency disease.
- Bone marrow transplantation has usually improved functional capacity in man.
- Controversial results concerning the rejection of skin of the thymus donor have been reported in previous transplant studies, using allogeneic mouse thymus donors.
- Further extensive testing is necessary to answer this intriguing question.
- Hair growth was not observed even after complete acceptance of thymus donor skin.
- Hair growth can be impaired even when rejection does not occur.
- Proliferation tests were not performed until after 5 d of culture.
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culture. Significant cell-mediated lympholysis (CML) (81%), however, was observed. These preliminary studies do not suggest that tolerance has occurred.

In this issue of tolerance to the donor antigens, it should be emphasized that there is a fundamental difference in our transplants and those employed in previous nu/nu reconstitution experiments. In contrast to previous work, we do not transplant significant numbers of viable donor thymocytes. This can be seen by viewing of the cultures (Figs. 1-3); we have also teased apart fragments after the culture period and searched for viable lymphocytes. Essentially none are found. This lack of transplanted thymocytes is of importance, as it seems reasonable to propose that the resident allogeneic thymocytes transplanted in noncultured tissues could in some way tolerize the host. It is important to know if thymic epithelium can in some way influence the histocompatibility reactivity of cells which differentiate within its confines. This possibility is suggested by the recent studies of Zinkernagel et al. (29, 30) who propose that H-2 restriction is determined by the histocompatibility markers of the thymus rather than those of the stem cells. In their studies, Zinkernagel et al. (30) found that T-cell precursors of strain A, maturing in a thymus of strain B, showed H-2 restriction in cytotoxic T-cell killing of vaccinia infected targets; i.e., B, but not A, targets were lysed. In a similar way, host T-cell precursors might acquire tolerance to the donor antigens from the thymic epithelium. Detailed studies addressing this point are presently underway.

The amount of thymic tissue seen in the kidney was surprisingly small. As examination of the thymus transplant was the last study performed, it is possible that larger grafts would have been found earlier in the first few months after grafting. There was no evidence that the T-cell function was waning in the immediate period before the terminal experiments, however. All animals were in excellent health and lymphocyte counts and Thy-1 cells remained at prior levels. No animal was examined before receiving a skin graft from the donor strain. It is assumed that the allogeneic thymus grafts would persist for an extended period even in the face of acquisition of normal T-cell capability, for transplants of allogeneic and xenogeneic CTF in normal immunocompetent hosts have been observed several months after transplant without any signs of inflammatory reaction. However, it is possible that when challenged with donor skin, tolerance would be abrogated due to the presence of macrophages and lymphoid elements in the skin graft, a phenomenon elucidated by Talmage et al. (31).

In the studies reported here, the allogeneic transplants were as effective as the syngeneic. Zinkernagel (32) has suggested that poor results in human reconstitution which utilized fetal thymic tissues could be related to his observation that hybrid cells differentiate recognition structures for self according to the H-2 of the thymus. Thus, hybrid (A X B) precursor cells maturing in a A thymus were found to be restricted in their cytotoxic potential to infected targets bearing A histocompatibility markers. In other words, the thymus controls the reactive capability of its progeny. Zinkernagel (32) extended his observations to suggest that T-B cooperation might also come under such restriction. By this logic, the H-2 of the thymus would have to be the same as the H-2 of the B cells to have effective helper function. Since allogeneic as well as syngeneic thymus resulted in restoration of IgA and IgG1 levels in addition to acquisition of thymic-dependent antibodies, T helper function was obtained regardless of H-2 disparity in our studies. Although we found normal CML, as yet we have
not utilized virus infected targets. Therefore, we do not have information on whether cultured thymic epithelium controls the H-2 restriction phenomenon as observed by Zinkernagel et al. (30). Nevertheless, we have observed in humans transplanted with allogeneic thymuses not only reconstitution of immunoglobulin levels of all three major classes, but the appearance of anti-viral antibodies in the wake of newly acquired viral infections from which the patients recovered (14).

No complications attributable to the graft were noted in these experiments. One theoretical problem could be the development of an autoimmune process. Jerne (33) proposed that the thymus acts as a filter for stem cells which would have self-reactive capability. In his theory, elimination of these autoreactive clones would prevent self-attack and would also serve to generate antibody diversity. Since allogeneic epithelium would screen out clones capable of attacking the donor, but not the host, a vigorous autoimmune process could result with the acquisition of T-cell competence. In one animal, a skin eruption consistent with chronic vasculitis was observed. Recurrent bouts of swollen joints were noted. However, in general, the animal seemed in as good health as his peers. His functional T-cell assessment was equivalent to the other transplanted animals; the skin eruption eventually faded and the joint swelling ceased.

Also of interest in this regard is the finding that only one of five nu/nu transplanted with CTF who were tested showed anti-nuclear antibodies, and this was very weak. In the control animals, the incidence was 50% (15), usually of 3+ intensity.

In a previous study, Willis and St. Pierre (34) showed that rat thymic monolayers could somewhat correct the deficiencies of thymectomized rats, but the grafts did not become lymphoid. They were adequately vascularized and remained intact in the host. The difference between their results and our studies, which use explants, may indicate that preservation of the original three dimensional structure, at least in part, is important in achieving lymphoid infiltration after establishment of the graft. We have not as yet employed monolayers in our experiments.

Monolayers have been shown by several workers to elaborate thymic factors capable of effecting some in vitro differentiation of precursor thymic cells (35–39). Similar results have been observed with supernates of our cultures (M. Borzy and R. Hong, unpublished observations). In some human studies, increase of thymopoietin-like material was seen after transplantation of cultured thymic epithelium (40). Similar measurements have not been performed as yet in our nude mouse recipients; however, the marked enlargement of the paired bodies is quite intriguing in this respect. Wortis et al. (9) found discrete paired bodies associated with cystic structures in nude mice. In most of our control untreated nude mice, as well as mice infused with blood, spleen or bone marrow, we have observed similar structures (M. Borzy and R. Hong, unpublished observations). They are quite small and found only upon careful dissection of the upper mediastinum. In five of nine nudes transplanted with cultured thymic epithelium but never in nontransplanted animals, these bodies were quite large and almost the size of a normal thymus. Grossly however, they were cystic and did not resemble a normal thymus. Upon histological section, there was no evidence of normal thymic structure or lymphoid accumulation (Fig. 7). The increase in size in the transplanted animals suggests a relationship of the paired bodies to thymic derivatives; they may very well represent vestigial thymic epithelial structures. It seems reasonable to suggest that the paired bodies were responsive to some humoral substance elaborated by the graft.
The degree of T-cell reconstitution in all transplanted animals was not complete. Total lymphocyte counts and counts of subpopulations attained approximately 50% of the values for normal BALB/c mice. In previous studies by others, thymus epithelium has been completely ineffective in achieving reconstitution (7). Loor and Hägg (7) found consistently poor repopulation of nude mice using epithelial transplants. Two methods of preparing epithelium were used by them, radiation or culture of thymuses for 1–2 wk in Waymouth's medium followed by RPMI-1640. It is not clear why our results differ from theirs, but subtle differences in culture technique or amount of tissue transplanted might explain the variance. Syngeneic noncultured thymuses have been shown to restore T-cell function to the same degree as we observed in our studies but allogeneic grafts are usually much less effective (6). In all cases, the in vitro responses are less than normals of the same strain (Table IV; references 6, 7). It may be that a dual defect exists in nude mice (stem cell as well as thymus), and T-cell responses of normal vigor cannot be obtained. In examining our transplants grossly at autopsy, it was not clear how large a thymus should be expected, but certainly, in no case was the thymus as large as the normal mediastinal structure. Therefore, the possibility exists that transplantation of much more cultured tissue might result in a greater degree of reconstitution. We cannot state what might be the equivalent of our grafts in terms of whole thymuses. Each animal received tissue derived from three newborns. A considerable amount of shrinkage occurs during the culture period, mostly as a result of thymocyte and macrophage depletion. It is unlikely that the epithelial cells replicate under the conditions of our culture. We have incubated [³H]thymidine with the cultures, teased the cells apart and prepared radioautographs. No thymidine incorporation was seen. Further, in other previous studies we showed that the chemotactic influence of thymic explants diminished considerably as the culture time was lengthened (15). It was also shown that lymphoid repopulation of transplanted tissue was poor when the pieces were cultured for longer periods (15). It may be that sufficient compromise of the epithelial potential has occurred during the culture period to require tissue from several donors to restore T-cell function to more normal levels. Recent observations by Talmage and Dart (41), showing that hyperbaric oxygen shortens the culture time to 4 d for thyroid tissue offer promise for improving our culture conditions.

If the cell-mediated lympholysis results indicate killer cell capability and are more a measure of the individual's ability to eliminate infectious agents controlled by the cell-mediated immune defenses than proliferative responses, then the actual degree of host protection produced by the graft might be nearly all that is necessary to survive in an unprotected environment. This observation would be consistent with results in human cultured thymus transplantation. In these cases, despite only modest proliferative T-cell responses (20% of normal), normal levels of immunoglobulins are seen and ability to recover from viral infections has been observed (13, 14). It might not therefore be necessary to regain full vigor of all the proliferative responses to have a clinical cure.

These studies show that CTF can reconstitute nude mice as effectively as intact newborn noncultured syngeneic thymus transplants. Allogeneic CTF transplants are as efficient as syngeneic. To extend these studies fully to clinical relevance in the human situation, further work considering use of multiple allogeneic donors, age of the donor, site of transplantation, etc. must be done. It must also be determined which
of the many forms of immunodeficiency are appropriately represented by the nude mouse.

Summary

Nine nude mice were transplanted with cultured thymic fragments derived from syngeneic (three recipients) or allogeneic (six recipients) sources. All transplanted mice survived for periods of up to 8-10 mo thereafter, at which time they were sacrificed. Weight gain had been progressive and the animals were in excellent health. Four nontransplanted littermates housed in the same cages died at the age of 4 mo. In the nontransplanted mice, the usual deficits of T and B cells were observed.

In transplanted mice, normalization of IgG1 and IgA levels as well as IgG antibodies to sheep erythrocytes and precipitating antibodies to rabbit serum occurred. Lymphocyte counts and Thy-1 bearing cells increased to approximately 50% of normal values. Proliferative responses to phytohemagglutinin and concanavalin A, mixed leukocyte reactivity, and cell-mediated lympholysis were variably restored from approximately 10-100% of normal. Attained responses were the same in recipients of syngeneic or allogeneic tissues and these, in turn, were equal or superior to responses measured in animals transplanted with whole noncultured thymuses.

Skin grafts from third party donors were vigorously rejected, whereas those derived from second party (allogeneic thymus donor strain) may have been accepted or slowly rejected.

Cultured thymic fragments, consisting primarily of epithelial elements, can effectively repair the thymic deficiency of nude mice. Experiments to date do not indicate that syngeneic tissues enjoy an advantage over allogeneic grafts in this restoration procedure.

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