Effect of Thymus Enclosed in Millipore Diffusion Envelopes on Thymectomized Hamsters

By F. M. Wong, R. N. Taub, J. D. Sherman and W. Dameshek

There is increasing evidence that the thymus has a central role in the development of the immune system.1,2 Surgical removal of the thymus in newborn mice and rats results in wasting, lymphocytopenia, and impaired immune responses.1,2 Previous studies from our laboratory demonstrated that similar effects were also produced in hamsters subjected to neonatal thymectomy.3,4

The existence of a humoral thymic factor which maintains immune responsiveness has been suggested by the prevention of wasting disease and loss of immune competence in thymectomized mice and rats implanted with cell-tight diffusion chambers containing thymus.5-10,59 These animals with implanted diffusion chambers, however, still showed evidence of lymphocyte depletion even though humoral antibody responses and homograft rejection were largely intact.5,6,8,10,11,59

The present report describes our studies on the effect of thymus and other tissues, enclosed in Millipore diffusion envelopes, on thymectomized hamsters. Part of these studies have been previously presented.12 Our results indicate that a humoral substance, apparently specific to the thymus, was capable of maintaining a normal humoral antibody response in thymectomized hamsters. This humoral substance was apparently produced by thymic epithelial-vascular cells; it failed to maintain normal levels of blood lymphocytes.

Materials and Methods

Hamsters: Randomly bred golden hamsters (Mesocricetus auratus) from our closed colony were used. These animals were quartered in an air-conditioned animal room and fed Purina Laboratory Chow and water ad lib. They were weighed at weekly intervals and weaned when 3 weeks.

Animals were thymectomized at 12–14 days of age by a technic previously described.3 Complete thymectomy was routinely verified at postmortem by gross inspection and histologic sections of suspect tissues. All animals with residual thymus were excluded.

Nonwasted thymectomized hamsters of both sexes were divided into 5 groups. Animals from each of 4 groups, when 3 weeks old, were implanted intraperitoneally with diffusion envelopes containing one of the following: an entire thymus from a 2-week-old donor, 30 million adult bone marrow cells, 50 mg. of adult spleen, or 10 mg. of adult kidney. The...
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remaining group of animals served as controls and was implanted with empty Millipore envelopes left open. One group of animals was not operated upon and served as normal controls. When it became evident that thymus enclosed in diffusion envelopes maintained a normal humoral antibody response in the thymectomized hamsters, an additional group was established. After 4-6 weeks implantation within the peritoneal cavity, the only cellular constituents of the thymus remaining in the diffusion envelope appeared to be epithelial- reticular cells. To see if these thymus epithelial- reticular cells could produce the same effect as the original whole thymus, some of these envelopes were removed after 4-6 weeks implantation, left unopened, and reimplanted into the peritoneal cavity of other 3-week-old thymectomized hamsters. This new group was then tested in the same way as the other groups.

Millipore Envelopes: Diffusion envelopes were constructed with Millipore* filter discs of 100 millimicra pore size using a technic developed by one of the authors (R. T.). Two 25 mm. filter discs were placed in apposition between two ordinary microscopic glass slides (Fig. 1). A bent-tip clamp (Lovelace) was used to apply focal pressure on the slides directly over the center of the discs. Acetone flowed between the slides and completely surrounded the filters, melting and fusing the edges but leaving the remainder of the filters intact. After about 20 seconds, the clamp was removed, the glass slides were gently separated, and the sealed envelope was lifted carefully from the slide with a razor blade. The sealed edge appeared as a thin transparent rim around the envelope. The envelopes were then cut in half, creating an opening, put in a petri dish, and sterilized in a dry oven for 24 hours at 60 C. Filling of the envelopes was carried out under sterile conditions. The open edge was placed between the slides, leaving the filled portion on the outside. Acetone was applied, as described above; the sealed envelope was then removed and immediately implanted.

Immunization: Animals were given two injections of 180 micrograms of human gamma globulin, fraction II (HGG)† in 0.1 ml. of a 1:1 mixture of complete Freund’s adjuvant (Difco) and saline. These injections were given into the footpads, 10 days apart, beginning 3-4 weeks after implantation with diffusion envelopes.

Serology: Blood for antibody titrations, serum and immunoelectrophoresis was obtained by cardiac puncture 10 days after the second injection of HGG. Antibody titer were determined by passive hemaggutinin. Twofold serum dilutions, starting with a 1:10 dilution, were made, using 0.2 per cent gelatin (Knox) as a diluent. Normal rabbit serum was not found suitable as a diluent because it contained naturally occurring anti-HGG activity. Sheep red cells were sensitized with a concentration of 2 mg. HGG per ml. of 2.5 per cent sheep cell suspension. The final red cell suspension was adjusted to a 2.5 per cent concentration with a Coleman spectrophotometer, after conversion to cyanmethemoglobin. All titrations were performed on the same day and appropriate controls were employed.

Blood Studies: Blood for hematologic studies was obtained from the footpad at the time of the first immunization. Hemoglobin, total white cell and differential counts were performed by previously described technic. Anti-hamster gamma globulin serum (Hyland) and anti-hamster serum, prepared according to Betts’ technic, were used for Coombs’ tests. Potency of Coombs’ sera was checked with tanned sheep cells passively sensitized with hamster serum.

Histology: Complete autopsies were performed on all hamsters sacrificed at 8-12 weeks of age and, when possible, on those dying spontaneously. Organs were fixed in 10 per cent formalin and stained with hematoxylin and eosin. Selected tissues were stained with Congo red.

RESULTS

Figure 2 shows the effect of thymus and other tissues enclosed in diffusion envelopes on the absolute blood lymphocyte count of 7-8-week-old hamsters.

*Millipore Filter Corporation, Bedford, Mass.
†Pentex Inc., Kankakee, Ill.
The lymphocyte count was significantly decreased in control thymectomized hamsters implanted with empty envelopes (p < .001). This reduction in lymphocyte count was not prevented by thymus (p < .025), spleen (p < .001), bone marrow (p < .01), or kidney (p < .01), in diffusion envelopes. Differences in lymphocyte counts between the groups receiving thymus and other tissues were not significant. There was no correlation between the lymphocyte count and the ability to produce humoral antibody.

Hemoglobin and total white blood cell counts were not decreased in any of
the groups. Direct Coombs' tests were negative on all animals. Serum and immunoelectrophoresis showed that gamma globulin levels were usually normal or increased in nonwasted hamsters bearing diffusion envelopes, and there was no significant differences between groups.

Figure 3 summarizes the antibody responses to HGG produced by hamsters implanted with Millipore envelopes. Control animals implanted with empty envelopes produce antibody titers generally less than 1:40. In contrast, animals implanted with thymus-containing envelopes usually produced normal amounts of antibody, with titers generally greater than 1:320.

Animals bearing envelopes containing kidney, spleen, or bone marrow were still impaired in their ability to produce humoral antibody. However, these animals were able to produce greater amounts of antibody than control animals implanted with empty envelopes.

The effect on humoral antibody production of reimplanted envelopes containing thymus, removed from the original host after 4–6 weeks, is shown in Figure 4. Hamsters with reimplanted envelopes and those implanted with the original thymus-containing envelopes produced comparable amounts of antibody to HGG. Absolute lymphocyte counts in both groups were low and were not significantly different.

Nonwasted hamsters implanted with envelopes gained weight and grew normally, and there were no significant differences between the groups regardless of the tissue contained within the envelopes. Some animals developed a wasting syndrome, but this was usually on a cage basis and appeared to have a random distribution among the groups.

All groups of thymectomized hamsters implanted with diffusion envelopes, regardless of the contents, showed a slight reduction in lymphocytes within the lymphoid tissues, after antigenic stimulation. An occasional animal showed normal or even hyperplastic lymphoid tissues with foci of splenic extramedullary hematopoiesis, and reticulum cell hyperplasia. Splenic Malpighian corpuscles were usually reduced in size, poorly organized, and lacked secondary follicle formation (Fig. 5). Lymph nodes generally showed altered architectures with poorly defined corticomedullary demarcations. Germinal centers and follicles were approximately normal in number but reduced in cellularity. In the majority of animals, plasma cells were either normal or increased in numbers (Fig. 6). Many animals showed a striking deposition of amyloid in the spleen and liver, which stained positively with Congo red (Fig. 7).

At autopsy diffusion envelopes were either free within the peritoneal cavity or encapsulated in a thin layer of fibrous tissue. Nonthymic tissues within the envelopes were completely necrotic after 4 weeks implantation. Thymic remnants, recovered from envelopes 4 weeks after implantation, contained 3–4 mm, gray-white, compact masses which were composed of necrotic debris and small clumps of cells resembling epithelial-reticular cells. In section, this cell appeared large and pale staining, with scanty cytoplasm, and had a large vesicular nucleus with a prominent nucleolus (Fig. 8). There was no evidence of cells traversing the millipore filter walls.
Fig. 3.—Antibody responses to HGG in animals implant with millipore-enclosed thymus and other tissues.

**Discussion**

The role of the thymus in immunogenesis is slowly unfolding. Four basic experimental methods have been used to illuminate this process: (1) thymus extirpation, (2) prevention or reversal of the effects of thymectomy, (3) phylogenetic experiments, (4) studies of ontogenesis. These studies all point to the thymus as a major primordium of the immune system.

Surgical removal of the newborn thymus produces 2 major effects, reduction in lymphocytes and impaired immune responsiveness. A wasting syndrome has also been described, but recent evidence suggests this may be a secondary phenomenon, due to infection.

Prevention of the effects of thymectomy can be accomplished by thymus grafts or injections of lymphoid cells. However, restoration or reconstitution of overtly wasted animals has not been successful with few exceptions, probably because by this time the animals are too ill with superimposed infections and possibly other derangements which may occur. Our decision to use nonwasted animals was influenced by this consideration.

Several mechanisms have been proposed to explain the participation of the thymus in immunogenesis. These include (1) cellular colonization of peripheral lymphoid tissues by cells from the thymus, (2) cellular migration into
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### Table: Effect of Reimplanted M.D.E. on Hamsters

<table>
<thead>
<tr>
<th>Reciprocal Anti-HGG TITERS</th>
<th>M.D.E. THYMUS</th>
<th>M.D.E. Reimplanted THYMUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs Lymphs/cm³</td>
<td>3400 ± 1800</td>
<td>2900 ± 1300</td>
</tr>
<tr>
<td>10-40</td>
<td>5/18</td>
<td>4/12</td>
</tr>
<tr>
<td>80-160</td>
<td>4/18</td>
<td>3/12</td>
</tr>
<tr>
<td>≤320</td>
<td>9/18</td>
<td>5/12</td>
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**Fig. 4.** HGG responses in thymectomized animals implanted with Millipore-enclosed thymus transferred from another thymectomized host.

**Fig. 5.** Lymphoid atrophy in thymectomized hamster.

depth, differentiation, and endowment with immunocompetence, (3) a thymic humoral factor. There is recent evidence that cellular migration from the thymus occurs. Histologic and electron microscopic studies have suggested a movement of thymic lymphocytes from thymic cortex to the bloodstream. Studies with the T6 marker chromosome or discriminant spleen assay, in thymus grafted mice, showed a consistent thymus-donor component in the host's lymphoid tissues, especially if stimulation of mitosis had been produced. In situ labeling of thymic lymphocytes with tritiated thymidine.
Fig. 6.—Lymph node, thymectomized hamster. Note increased numbers of plasma cells.

Fig. 7.—Amyloid deposition in hamster spleen following thymectomy.

showed seeding of small numbers of small lymphocytes into all lymphoid tissues.72

There is some evidence that cells enter the thymus, where they may differentiate and become immunocompetent. After about 2 weeks, thymus grafts are repopulated by host cells,19,28,33 and on retransplanting the thymus graft to a second host, there is a second repopulation with the new host's cells.28 Regen-
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Fig. 8.—Section of a hamster thymus from a diffusion envelope after 4 weeks implantation within the peritoneal cavity, 800 ×. Many large, pale staining epithelial-reticular cells each with a large vesicular nucleus and prominent nucleolus remain. Insert shows one of these cells under higher magnification, 1600 ×. Thymic lymphocytes are absent. Many dark staining clumps of necrotic debris are present.

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sponsiveness in these animals was largely normal. Our observations are in agreement with these findings. We found that thymectomized hamsters, implanted with thymus in diffusion envelopes, showed a significant lymphocytopenia even though humoral antibody production was normal. In contrast, intact thymus grafts appeared to produce both normal lymphocyte levels and normal immune responsiveness in thymectomized animals.23,30,31,40

It would appear that the intact thymus was capable of maintaining normal lymphocyte levels, but neither thymocytes nor the humoral factor from diffusion chamber enclosed thymus, separately, had this ability. The mechanism by which the intact thymus produces this effect is unknown. The intact thymus may survive better and produce a greater quantity of humoral factor than a diffusion chamber enclosed thymus. However, the survival of diffusion envelope enclosed thymus did not appear seriously impaired, as judged by their ability to maintain a normal humoral antibody response in our hamsters. On the other hand, it may be that both cells from the thymus and a humoral thymic factor are necessary to maintain normal lymphocyte levels. Alternatively, the intact thymus may provide a special environment for the differentiation and proliferation of lymphocytes. The diffusion chamber would prevent the passage of cells into or out of the thymus, and this may account for the lymphocytopenia observed in these animals. Furthermore, either mechanism could also explain why thymocytes or other lymphocytes, in high doses, did not have the ability to maintain normal lymphocyte levels. These infused cells may require a proliferative stimulus provided by the intact thymus.

In thymectomized animals with lymphocytopenia, a fraction of lymphocytes remains which may remain stable for periods of at least 4–12 months, but not always.1 This persistent fraction of lymphocytes can be explained by postulating the existence of two different lymphocyte populations. These may represent long and short-lived lymphocytes, with the former continuing to survive after thymectomy. Or, as previously postulated, the two populations may represent lymphocytes produced by two different sources, the thymus and a nonthymic source.41 It has been suggested that the nonthymic source may be the bursa in chickens,43 the bone marrow in mice,35 and the tonsils in man.2 The remaining population in thymectomized, lymphocytopenic animals shows a reduction in small lymphocytes.5,41,57,58 These remaining lymphocytes may account for the ability to produce humoral antibody and the plasmocytosis and hypergammaglobulinemia observed in our hamsters.

The ability of thymus enclosed in diffusion envelopes to maintain a normal humoral antibody response in hamsters suggests a humorally mediated mechanism. Similar results, indicating the existence of a humoral substance from the thymus which was not found in spleen or lymph node, have been reported in mice and rats.5,7,9,11,59 However, we found that adult kidney, spleen, or bone marrow, enclosed in diffusion envelopes, enhanced humoral antibody production slightly. In this connection, saline filled, intraperitoneal diffusion chambers were also found to have an adjuvant effect on the humoral antibody response if the antigen was given intraperitoneally, but not if it was given at a distant
subcutaneous site. It is conceivable that our tissue-filled envelopes had an adjuvant effect even though our antigen was given into the footpad. It is also possible that breakdown products from necrotic tissues may have enhanced humoral antibody production. On the other hand, the possibility that these nonthymic tissues enhanced antibody production by a mechanism similar to the thymus cannot be excluded. However, animals receiving nonthymic tissues in diffusion envelopes were still impaired in their ability to produce humoral antibody. In contrast, animals receiving thymus-containing envelopes produced normal amounts of antibody.

The cellular source of the thymus humoral factor appears to be the thymic epithelial-reticular cell. As previously described by Downey and Smith, this cell, in section, has a round, irregular, or deeply grooved, extremely pale nucleus with a sharply defined spherical nucleolus. The amount of cytoplasm varies and shows very little affinity for any stain. Osoha attributed the thymus humoral substance to cells resembling thymic epithelial-reticular cells which survived in diffusion envelopes up to 10 weeks after implantation. Our results are in agreement with this thesis. Envelopes, removed and reimplanted into other animals after 4 weeks, were devoid of thymic lymphocytes but contained large cells resembling thymic epithelial-reticular cells. These envelopes were found to exert an effect on the humoral antibody response comparable to the original envelopes containing whole thymus.

Two major effects of the thymic humoral factor have been proposed: stimulation of lymphopoiesis and induction of immunocompetence of lymphocytes. These effects appear to be separate, as suggested by diffusion chamber implanted animals with intact immunity but depleted lymphocytes. It is possible that these are two different humoral thymus factors: a lymphocytosis stimulating factor and a competence inducing factor. There may be, in addition, other humoral thymus factors, producing increased susceptibility of thymectomized mice to lymphochoriomeningitis infection, retardation of skin tumors in mice, and inhibition of humoral antibody production. These seemingly widely separated effects, however, may be due to a single humoral factor with several different effects, having as a common denominator the production of a single alteration in a metabolic process of the lymphocyte.

Amyloid deposits in the spleen and liver were frequently observed in our thymectomized, diffusion envelope implanted hamsters which had been immunized with HGG and complete Freund's adjuvant. In contrast, an equal number of similarly immunized, intact, control hamsters rarely showed amyloid deposits, although amyloidosis has been previously observed by others in normal hamsters. Amyloidosis has been reported in mice and rabbits following thymectomy. In rabbits, amyloidosis was regarded as part of an autoimmune process initiated by thymectomy and appendectomy. It is possible that thymectomy was the cause of amyloidosis in our hamsters, however, other stimuli, including complete Freund's adjuvant, and possibly the diffusion envelopes themselves may have played a role. Coombs' tests and histologic sections revealed no direct evidence of autoimmune disease in our thymectomized hamsters.
Summary

Neonatally thymectomized hamsters implanted with diffusion envelopes containing thymus showed partial prevention of the effects of thymectomy. A normal humoral antibody response was maintained in these animals, although a significant lymphocytopenia remained. This suggests that the thymus participates in immunogenesis by a humoral mechanism and possibly by a cellular mechanism as well, which was blocked by the diffusion envelope. Thymectomized hamsters implanted with diffusion envelopes containing adult spleen, bone marrow or kidney were still impaired in their ability to produce humoral antibody, although these tissues appeared to enhance antibody production. The thymus humoral factor appeared to be specific to the thymus. Reimplanted diffusion envelopes, containing large thymic cells resembling epithelial-reticular cells, were used to show that these cells produced the thymus humoral factor.

Summario in Interlingua

Hamsters thymectomisate neonatalmente e subjicite al implantation de involoppes de diffusion continente thymo monstrava un prevention partial del effectos de thymectomia. Un normal responsa de anticorpore humoral esseva mantenite in iste animales ben que un significative lymphocytopenia persisteva. Isto suggestiona que le thymo participa in le immunogenese per un mechanismo humoral e possibilemente etiam per un mechanismo cellular le qual esseva blocate per le involoppe de diffusion. Thymectomisate hamsters subjicite al implantation de involoppes de diffusion continente adulte splen, medulla ossee o ren remaneva defective in lor capacitae de producere anticorpore humoral ben que le tissus mentionate pareva promover le production de anticorpore. Le factor humoral de thymo pareva esser specific pro le thymo. Reimplantate involoppes de diffusion continente grande cellulas thymic resimilante cellulas epithelio-reticular esseva usate pro monstrar que iste cellulas produceva le factor humoral thymic.

References

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