American Journal of Pathology, Vol. 133, No. 1, October 1988

Complete Sequential Regeneration of Graft-vs.-Host-Induced Severely Dysplastic Thymuses

Implications for the Pathogenesis of Chronic Graft-vs.-Host Disease

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This study presents the sequential morphologic regeneration of graft-*vs.*-host (GVH) -induced dysplastic thymuses in long-term survivors of GVH reactions. GVH reactions were induced in adult C57BL/6xAF$_1$ (B6AF$_1$) hybrids by injecting 20×10^6 A strain parental lymphoid cells (PLC). Starting on day 30 after GVH induction, five to ten animals were randomly selected from a pool of GVH-reactive mice and killed at various times. Each animal was tested for thymic histology and T cell functions. Thymuses taken on day 30 after GVH induction displayed severe dysplasia as characterized by lymphocytic depletion, complete effacement of cortico-medullary demarcation, and reduction and total loss of medullary epithelial cells or both. Starting by days 60–70 after GVH induction, at least four stages of thymic regeneration were identified. Day 60–70 thymuses displayed cortical regeneration and the reappearance of cortico-medullary demarcation. The medulla of these thymuses, although containing dark individual epithelial cells and numerous lymphocytes, was devoid of pale epithelial cells (stage 1). The medulla of thymuses on day 100 after GVH induction displayed a few sparcely distributed pale epithelial cells and numerous lymphocytes as well as dark epithelial cells (stage 2). The medulla of thymuses examined 130 days after GVH induction displayed numerous pale individual epithelial cells and a few pale epithelial cell clusters. Such thymuses also showed a reduction in the number of medullary lymphocytes (stage 3). Finally, the medulla of thymuses 150–160 days after GVH induction displayed numerous pale epithelial cell clusters and Hassall's bodies. These thymuses were indistinguishable from normal adult thymuses (stage 4). All of the animals tested up to day 130 after GVH induction showed no significant T cell function. Animals displaying stage 4 of thymic regeneration showed significant proliferative responses to T cell mitogen, concanavalin A (conA), and six of ten animals also displayed a few plaque forming cells (PFC) to sheep red blood cells (SRBC) in their spleens. Furthermore, all animals (10 of 10) killed on day 180 after GVH induction displayed significant T cell functions. These studies indicate that cortical regeneration precedes medullary regeneration and that during medullary regeneration, morphologically distinct types of epithelial cells appear in a definite sequence. Furthermore, complete medullary regeneration takes approximately 90–100 days after the regeneration of the cortex and the reappearance of a cortico-medullary demarcation. Moreover, T cell functions recover gradually only after the thymic medulla has regenerated. The implications of this long process of thymic regeneration on immune function and on the development of chronic GVH disease are discussed. (Am J Pathol 1988, 133:39–46)

THE THYMUS HAS an essential role in the production and maturation of T lymphocytes. This thymic function is thought to be achieved by physical contact between the thymic epithelial cells and lymphocytes and by the elaboration of factors by thymic epithelial cells.[1–3]

Supported by the Medical Research Council of Canada and the National Cancer Institute of Canada.

Accepted for publication May 13, 1988.

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The authors have reported previously that graft-*vs.*-host (GVH) reactions in adult non-X-irradiated F_1 hybrids cause severe thymic dysplasia.[4,5] Thymic dysplasia has been reported by other workers in X-irradiated mice[6] and rats[7-9] that received allogeneic spleen and bone marrow cells (allogeneic radiation chimeras [ARC]) as well as in human bone marrow recipients who develop GVH disease.[10,12] Varying degrees of thymic dysplasia (generally attributed to a developmental arrest) have also been described in patients with severe combined immunodeficiency (SCID).[13,14] It has been suggested that the thymic dysplasia observed in some SCID patients may be due to the initiation of a GVH reaction by maternal lymphocytes that gain entry into the fetus and, through immunologic mimicry, escape elimination.[13] Moreover, thymic lesions akin to dysplasia have also been observed in patients with the acquired immune deficiency syndrome (AIDS).[15]

GVH reactions also cause severe prolonged T cell immunodeficiency.[16-19] The authors have reported previously that this T cell immunodeficiency is associated with thymic dysplasia.[16,17] More recent studies have demonstrated that GVH-induced thymic dysplasia causes a maturational arrest of interleukin-2 (IL-2) producing T helper cells.[18,19] It has been suggested that the severe prolonged T cell immunodeficiency observed in allogeneic radiation chimeras and human recipients of bone marrow who develop GVH disease may be due to thymic dysplasia, thymic hypofunction, or both.[20-22]

Interestingly, in long-term human survivors after bone marrow transplantation, cells expressing markers for different T cell subpopulations reappear and varying degrees of T cell functional restitution are observed.[23-29] Because the thymus has a central role in the production and maturation of T cells,[1,2] it is conceivable that the reappearance of various T cell subpopulations and T cell functional restitution may reflect different degrees of thymic regeneration. Furthermore, several workers have suggested that the acquisition of a normal thymic architecture (and function) may be essential for the development of stable chimeras.[20,21,30]

This study investigated the sequence of thymic histologic regeneration and its effect on T cell functions after severe dysplasia. The findings indicate that complete thymic regeneration appears to occur in distinct stages. These experiments establish that severely dysplastic thymuses resulting from the GVH reaction can indeed regenerate and such regeneration occurs in a definite sequence. The regeneration of the cortex and the reappearance of cortico-medullary demarcation precede the regeneration of the medulla. Moreover, medullary regeneration takes place in distinct stages. These histologic observations are discussed in relation to T cell function and the pathogenesis of chronic GVH disease.

Materials and Methods

Animals

Female mice of inbred strains A (H-2^a) and C57BL/6(B6)xAF_1 (H-$2^{a/b}$) ($B6AF_1$) were used. The recipient mice were between 10 and 14 weeks old at the time of GVH induction. Donor mice were between 12 and 16 weeks old. All animals were bred and maintained in our animal colony.

GVH Reaction Induction

Single cell suspensions were prepared in Hank's balanced salt solution from pooled spleens and lymph nodes from A strain mice. Each $B6AF_1$ recipient was injected intravenously with 20×10^6 donor lymphoid cells.

Thymic Histology

Thymuses from normal and GVH-reactive mice at different days after GVH induction were excised and fixed in 5% formalin. Paraffin-embedded sections were cut, stained with hematoxylin-phloxine-saffron (HPS), and examined histologically in coded fashion.

Assessment of T Cell Function

T cell function was assessed by measuring the proliferative ability (^{3}H-thymidine incorporation) of splenocytes in response to the T cell mitogen, concanavalin A (conA) and by measuring the plaque-forming cell (PFC) response to sheep red blood cells (SRBC). Both the mitogen assay and the PFC assay have been described in detail previously.[16-19]

Results

In preliminary experiments it was determined that 20×10^6 A strain lymphoid cells was the critical dose that induced severe thymic dysplasia yet allowed more than 90% of the animals to survive for longer than 6 months. A lymphoid cell dose of 10×10^6 failed to induce severe thymic dysplasia, whereas a cell dose of 30×10^6 resulted in greater than 90% mortality by day 40 after GVH induction.

GVH reactions were induced in a large group of $B6AF_1$ mice. On various days after GVH induction six to ten animals were selected at random and killed.

Such a protocol enabled examination of the status of the thymus in the same group of GVH-reactive mice.

Table 1 shows that all animals selected at day 30 after GVH induction displayed severe thymic dysplasia. However, starting from days 60–70 after GVH induction various stages of thymic regeneration were observed and, by days 150–160, all mice displayed normal thymic architecture. Because GVH reactions were induced by the same cell suspension and at the same time in all animals examined, it would appear that, on a statistical basis, all animals displayed severe thymic injury and, if left for an appropriate time, would undergo complete recovery of thymic structure. By days 150–160 after GVH induction the GVH thymuses were indistinguishable from normal thymuses. The morphologic changes observed for each stage of regeneration were consistent and are discussed below.

Table 1—The Status of Thymic Architecture of B6AF$_1$ Mice on Different Days After GVH Induction

Days after GVH induction	Number of animals	Status of thymus*
30	8	Severe dysplasia
60–70	9	Stage 1, regeneration
100	6	Stage 2, regeneration
130	6	Stage 3, regeneration
150–160	10	Stage 4, regeneration

* See text for a description of the different stages of regeneration.

Severe Lesions (Day 30 after GVH Induction)

Severe thymic dysplasia was characterized by a drastic decrease in thymic size, a complete loss of cortico-medullary demarcation, reduction of the number of cortical lymphocytes, and a marked reduction or total loss of medullary epithelial cells and Hassall's bodies (Figure 1A,B).

Stage 1 of Regeneration (Days 60–70 After GVH Induction)

This stage featured cortical repopulation and reappearance of a distinct cortico-medullary demarcation (Figure 2A). The medulla of such thymuses contained a much greater number of lymphocytes than a normal thymic medulla (Figure 2A). Close examination of the medulla showed dark individual epithelial cells that could be distinguished easily from lymphocytes; yet large pale individual epithelial cells, epithelial cell clusters and Hassall's corpuscles were not detected (Figure 2B).

Stage 2 of Regeneration (Day 100 After GVH Induction)

At this stage dark individual epithelial cells were readily observed throughout the medulla. Only a few sparcely distributed, large, pale epithelial cells were visible. No Hassall's bodies were observed. The medulla still contained a large number of lymphocytes (Figure 3A,B).

Stage 3 of Regeneration (Day 130 After GVH Induction)

These thymuses displayed a reduction in the number of medullary lymphocytes (Figure 3C) and a greater number of pale epithelial cells scattered

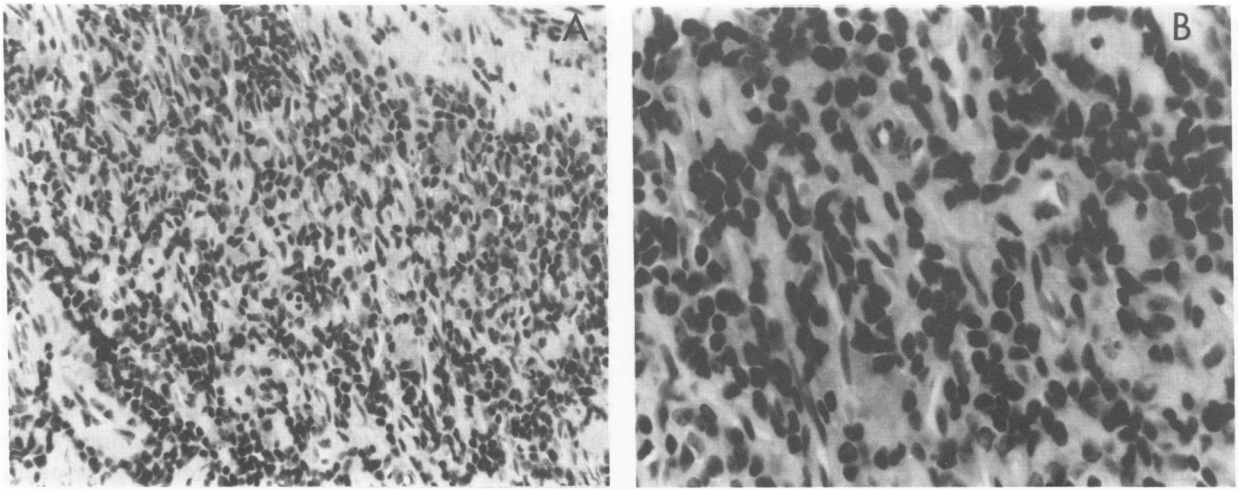

Figure 1—Thymus showing severe dysplasia. **A**—Note the absence of cortico-medullary demarcation and lymphocytic depletion. (×200) **B**—The medulla of the same thymus as in Figure 1A. Note the lack of pale medullary epithelial cells and Hassall's bodies. (×400).

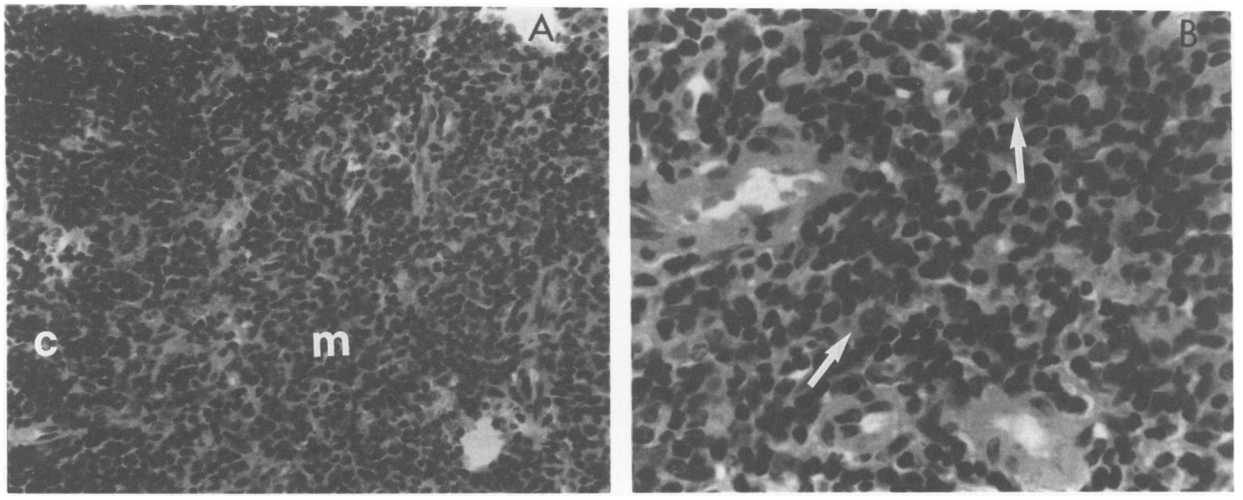

Figure 2—Thymus showing stage 1 of regeneration. **A**—Note the repopulation of the cortex (c) and reappearance of cortico-medullary demarcation. Also note the presence of numerous lymphocytes in the medulla (m). (×200) **B**—The medulla of the same thymus as in Figure 2A. Note the presence of dark individual epithelial cells (arrow) and absence of pale epithelial cells. (×400)

throughout the medulla than at stage 2 (Figure 3D). No Hassall's bodies were evident.

Stage 4 of Regeneration (Days 150–160 After GVH Induction)

The medulla of these thymuses (stage 4) displayed widely scattered Hassall's bodies and pale epithelial cell clusters (Figure 3E,F). They could not be distinguished from normal adult thymuses.

On a given day after GVH induction, all the thymuses examined displayed essentially the same stage of regeneration (Table 1), but there was some slight variation between animals. These stages of thymic regeneration have been observed in $B6AF_1$ mice injected with 30×10^6 C57BL/6 lymphoid cells as well; however, in this combination complete thymic regeneration was observed by days 120–130 after GVH induction (Ghayur T, Seemayer TA, Lapp WS, unpublished observations).

T Cell Function During Various Stages of Thymic Regeneration

Table 2 shows the conA responsiveness and the PFC response to SRBC (which is a T cell-dependent B cell response) of B6AF1 mice on different days after GVH induction. The data are from the same animals as those reported for thymic regeneration (Table 1). The results in Table 2 show that the GVH-reactive mice remain completely suppressed for both these T cell functions up to day 130 after GVH induction, ie, stage 3 of thymic regeneration. Table 2 further shows that by days 150–160 after GVH induction (a time when the thymuses display a near-normal morphology), significant T cell proliferative responses were observed, but no significant recovery of the PFC response was observed. However, by day 180 after GVH induction all the animals examined (10 of 10) demonstrated significant responses to both conA and SRBC. The results presented in Table 2 suggest that the immune functional recovery is gradual and is observed only after the thymic medulla has regenerated.

Discussion

These experiments illustrate that a severely dysplastic thymus stemming from a GVH reaction can regenerate a near-normal morphology. Furthermore, at least four different stages of regeneration have been identified that appear to follow a consistent pattern. During early regeneration, cortical repopulation and reappearance of cortico-medullary demarcation were initiated by days 60–70; complete medullary regeneration, however, was not observed until days 150–160. Thus, complete medullary regeneration took up to 3 months longer after cortical regeneration. Moreover, morphologically distinct epithelial cells were observed at different stages of medullary regeneration. Whether these epithelial cells represent different stages of maturation/differentiation or are separate cell populations is not known. These studies illustrate that with time and the appropriate conditions, GVH-dysplastic thymuses can regenerate and regain a normal architecture. The results presented in this study also show that the T cell function recovers gradually only after the thymus has regenerated completely, suggesting a close association between thymic medullary regeneration and the recovery of T cell functions after GVH induction.

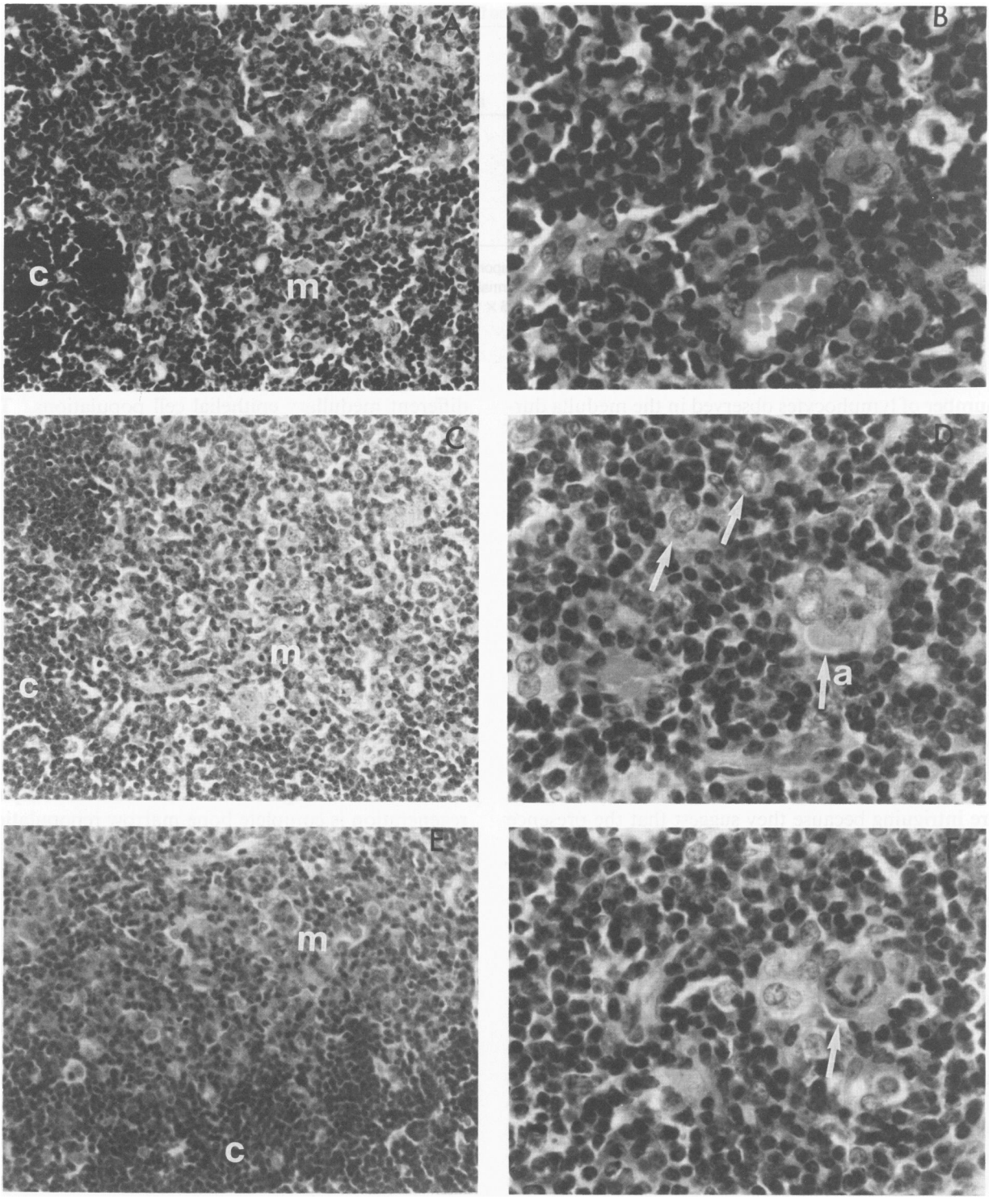

Figure 3—Thymuses showing various stages (2–4) of medullary regeneration. **A**—Stage 2 of regeneration. Note the presence of a distinct cortico-medullary demarcation and lymphocytes in the medulla (m). (×200) **B**—The medulla of the same thymus as in Figure 3A. Note the presence of numerous dark individual epithelial cells (arrows) and a few pale epithelial cells. (×400) **C**—Stage 3 of regeneration. Note the decline in the number of medullary lymphocytes. (×200) **D**—The medulla of the same thymus as in Figure 3C. Note the presence of numerous pale individual epithelial cells (arrow) and a epithelial cell cluster (arrow a). No Hassall's bodies were observed. (×400) **E**—Stage 4 of regeneration. (×200) **F**—Medulla of the same thymus as in Figure 3E. Note the presence of a nicely formed Hassall's body (arrow). Thymuses at this stage of regeneration were indistinguishable from normal thymuses. All sections were stained with hematoxylin-phoxine-saffron.

Table 2—Splenic ConA and PFC Response to SRBC of $B6AF_1$ Mice During Various Stages of Thymic Regeneration

Days after GVH induction	Stage of thymic regeneration	No. of animals	Immune functions (% of normal response ± S.E.)*	
			ConA	PFC/Spleen
30	Severe dysplasia	8	8.3 ± 2.4	0.0
60–70	Stage 1	9	10.9 ± 3.3	0.0
100	Stage 2	6	19.1 ± 6.4	0.0
130	Stage 3	6	15.8 ± 5.6	0.0
150–160	Stage 4	10	65.4 ± 9.0	1.5 ± 0.6
180	Stage 4	10	83.6 ± 7.9	30.2 ± 9.5

* Percent of normal response was calculated by using ConA and PFC responses of normal $B6AF_1$ mice in the same experiment. Three to five normal age and sex matched mice were used/day. The range of the mean values for normal ConA responses was 167 ± 8.2 to 191.9 ± 9.6 cpm ± SE × 10^{-3}. The range of mean values for normal PFC/spleen was 64.5 ± 6.2 × 10^{-3} to 116.1 ± 6.6 × 10^{-3}.

An interesting aspect of these observations is the number of lymphocytes observed in the medulla during different stages of regeneration. During stage 1 of regeneration, although a distinct cortico-medullary demarcation was present, a large number of lymphocytes were present in the medulla (unlike the normal medulla). At this stage (stage 1) the thymic medulla contained only sparce numbers of dark individual epithelial cells and was completely devoid of pale epithelial cells. Moreover, during stage 2 of regeneration, although a few sparsely distributed pale medullary epithelial cells appeared, the medulla still contained a large number of lymphocytes (Figure 3A,B). The concentration of medullary lymphocytes declined only when the number of pale epithelial cells increased and the latter were present throughout the medulla (stage 3 of regeneration, Figure 3C,D). These observations are intriguing because they suggest that the presence of cortico-medullary demarcation may not be sufficient to control the entry into and proliferation of lymphocytes in the medulla but that other factors associated with pale epithelial cells regulate the numbers of lymphocytes in the medulla.

Several workers have suggested that after BM transplantation in humans, the regenerating immune system recapitulates ontogeny.[20] Because T cell production and maturation are dependent on a normally functioning thymus, it would appear that regeneration of thymic architecture, after GVH-induced dysplasia might also recapitulate ontogeny. It has been suggested in rodents[34] and birds[35] that during ontogeny the entry of cells into the thymic anlage provides the stimulus for the differentiation and development of the medulla. It has been shown similarly, in sheep that the repopulation of thymic cortical regions precedes medullary development during ontogeny.[36] In these models the sequential appearance of different medullary epithelial cells is not clear. In contrast, in humans the time when the different medullary epithelial cells appear during fetal development has been described in detail by morphologic studies and by employing monoclonal antibodies directed against different medullary epithelial cell populations.[3] The thymic medullary development in humans[3] and the medullary regeneration after GVH-induced thymic dysplasia in mice have many common features (at least morphologically). A comparison of the regeneration of GVH dysplastic thymuses with the development of the human fetal thymus suggests that the regenerating GVH dysplastic thymus does indeed recapitulate ontogeny.

The authors have observed that in the non-X-irradiated F1 hybrid model of GVH disease, the initiation of thymic regeneration (cortical repopulation) coincides with spontaneous complete repopulation of the bone marrow following its severe depletion (Ghayur T, unpublished observations).[31] Therefore, it is possible that the limiting factor in the initiation of thymic regeneration is complete bone marrow repopulation, suggesting that the cells repopulating the thymus are of marrow origin. Given the dependence of thymic regeneration on marrow-derived cells, it is plausible that at least four factors have a critical role in determining the time required for the dysplastic thymus to regenerate: 1) genetic disparity between the donor and host; 2) the donor:host stem cell ratio repopulating the system; 3) the intrinsic regenerative capacity of the host thymus, which may be related to the age of the recipient, and 4) mechanism(s) generated during the initial acute GVH reactions that may play an important role in maintaining the lesions. One such radiosensitive mechanism has been reported previously.[37]

The authors have reported previously that the loss of thymic medullary epithelial cells after GVH induction is associated with a maturational arrest of T helper cells.[16–19] It is conceivable that the time required for complete medullary regeneration also may determine the duration of GVH-associated T cell immunodeficiency. The results presented in this study show that T cell function recovers gradually after the thymic medulla has regenerated. It is of interest to note that T cell proliferative responses recover earlier

than T cell-dependent B cell responses. The reasons for the delay in recovery of the PFC response are not known; however, it is possible that either more time is required for the regenerated thymus to regain functions that may be essential for the generation of T cells involved in T–B cell cooperative responses or more time may be required to accumulate a critical number of immunocompetent T cells in the periphery to mount a detectable PFC response to SRBC. It is also plausible that the defect may not be in T cells, but rather in some other cell population, ie, B cells or macrophages. The authors are investigating these possibilities currently.

The clinical relevance of the long process of thymic regeneration is not clear at this time, but it has been suggested that human thymic damage caused by GVH disease also is reversible.[11] Much evidence suggests that T cells learn class II restriction and self tolerance in the thymus as a result of an interaction with medullary epithelial cells. It is plausible that the long period of time required for medullary regeneration after GVH induction may provide an environment in which T cells develop without "learning" self–nonself discrimination, resulting in the production of autoimmune cells as the medulla recovers. Such autoreactive cells may contribute to chronic GVH disease, which is generally regarded as an autoimmune phenomena. Thus, the prolonged regeneration of the thymus after an acute GVH reaction may be a major contributing factor to the chronic GVH disease state. It is of interest that Parkman[38] has shown that all T cell clones isolated from mice undergoing chronic GVH reaction displayed autoreactivity against class II antigens and secreted collagen-stimulating leukokines. The hypothesis that the cells emigrating from a thymus devoid of a functional medulla may be the effectors of chronic GVH disease is similar to that proposed by other authors[20,30,32] for the generation of effectors of chronic GVH disease in rodents treated with cyclosporin A (CSA). It should be noted that CSA treatment (as GVH reaction) severely reduces the content of both the medullary epithelial cells and also decreases the medullary expression of class II antigens.[30,33]

In conclusion, thymic morphologic regeneration after GVH reactions occurs in a sequential pattern. The sequential thymic regeneration of an adult animal may be a powerful tool for study of the role of thymic elements in the maturation of T cells or in the generation of stable chimeras, as well as provide insight into the role of thymic stromal elements at different stages of T cell ontogeny.

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Acknowledgment

The authors thank Ailsa Lee Loy and Rosemarie Siegrist-Johnstone for careful technical assistance, Letti Pegorari for preparation of the histologic sections, and Sandra James for typing the manuscript.