Brief Definitive Report

ROLE OF THE THYMUS IN NATURAL TOLERANCE TO AN AUTOLOGOUS PROTEIN ANTIGEN

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The thymus is known to play a key role in self vs. non-self recognition (1). For example, thymic grafts have been shown to induce tissue transplantation tolerance in allogeneic models in both mice and birds (2–4). The thymus is also the site of tolerance induction to self MHC (5), but whether tolerance to autologous protein antigens originates in the thymus is unknown. Analysis of self tolerance has been hampered not only by the lack of suitable experimental systems, but also by the presence of autoantigens, which renders the detection of either cellular or humoral immunity difficult. We have examined the role of the thymus in natural tolerance to a physiologic protein antigen (the fifth component of mouse complement, C5) by taking advantage of two congenic strains of mice that differ only in the presence or absence of C5. Our experiments indicate that C5-deficient ($C5^-$) mice grafted with thymus from C5-sufficient ($C5^+$) mice failed to make humoral antibody to C5, suggesting that the transfer of thymus had induced tolerance. To further establish that tolerance was acquired in the thymus, we were able to adoptively transfer the state of tolerance by lymphoid cells from the $C5^-$ mice grafted with $C5^+$ thymus into irradiated $C5^-$ hosts. These results in mice with identical MHC appear to be the first to demonstrate that natural tolerance to self-protein antigen is "learned" in the thymus. This observation may have both fundamental and clinical significance.

Materials and Methods

Animals. Male 6–8-wk old B10.D2 OSN/J, B10.D2 NSN/J, and A/J, and newborn (1–3-d-old) B10.D2 OSN/J and B10.D2 NSN/J mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and maintained at The Center for Blood Research animal facilities. Animals to be irradiated were prepared as previously described (6).

Antigens. Murine C5 for immunization was prepared from the acid euglobulin fraction of B10.D2 NSN serum as previously described (6) at a dose of 50 μg per mouse in CFA (Difco Laboratories Inc., Detroit, MI), intraperitoneally. OVA (Sigma Chemical Co., St. Louis, MO) dose was also 50 μg per mouse in CFA, intraperitoneally.

Thymectomy and Neonatal Thymic Grafting. Adult mice were thymectomized using standard surgical techniques. Neonatal mice were killed by ether anesthesia and their thymic lobes were grafted subcutaneously under the left axillae of recipient mice. Thymectomy and engraftment were confirmed histologically.

Radiation Transfer Protocols. B10.D2 mice were lethally irradiated with 780 rad in a divided dose as previously described (6).

This work was supported in part by National Institutes of Health grants AI-07306, AA-07519, AM-7448, and AM-20582.

Cell Preparations for Transfers. Bone marrow was obtained from B10.D2 OSN mice, treated with anti-Thy-1.2 and guinea pig complement and 10^7 cells injected intravenously into irradiated recipient mice. Nonadherent (NA) spleen cell suspensions were prepared as previously described (6) and 7×10^7 cells were injected intravenously into recipients.

Assay of Mouse C5 and Anti-mouse C5. These assays were done as previously described (6).

Assay of Mouse Anti-OVA. Anti-OVA antibody response was measured by ELISA at a sera dilution of 1:1,000.

Statistical Analysis. For C5 inhibition levels, C5 levels, and anti-OVA responses, arithmetic means and SDs were calculated. Individual positive responses were determined as those values above the mean plus two SDs of the control group. Groups were compared by student's *t* test.

Results

Adult $C5^-$ (B10.D20SN) hosts were thymectomized, grafted with neonatal thymus from either $C5^-$ or $C5^+$ (B10.D2NSN) donors, irradiated, and reconstituted with anti-Thy-1.2 + C-treated bone marrow from $C5^-$ donors. In both groups the serum C5 level measured by a hemolytic assay was nondetectable. In preliminary experiments, $C5^-$ mice grafted with neonatal $C5^+$ thymus and immunized with C5 in CFA 3 wk after reconstitution showed an initial weak antibody response to the primary (1°) immunization that diminished after secondary (2°) and tertiary antigen challenge as assayed by inhibition of C5-dependent hemolysis. In a second experiment, $C5^-$ mice grafted with $C5^+$ thymus were challenged with C5/CFA 2 mo after reconstitution and their antibody response was measured (Fig. 1). Before immunization, serum C5 levels were undetectable in $C5^-$ mice with either $C5^-$ or $C5^+$ grafts (data not shown). Control $C5^-$ mice grafted with autologous thymus responded in the 1° response, although slightly less than intact $C5^-$ controls; in the 2° response, both of these groups responded equally well. In contrast, $C5^-$ mice grafted with $C5^+$ thymus failed to respond both in the 1° and 2° response. The results suggest that the thymus grafts from $C5^+$ mice induced tolerance to C5 in the $C5^-$ hosts.

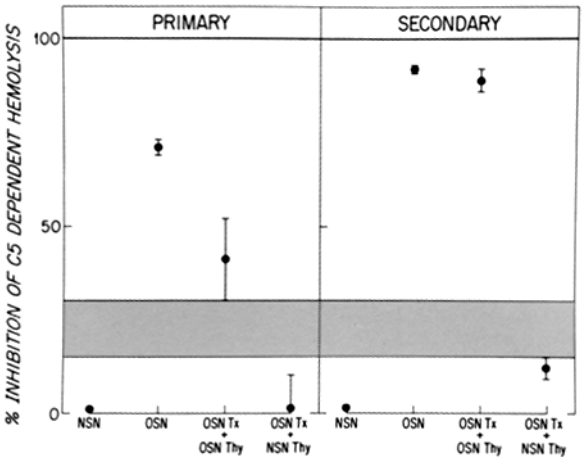

FIGURE 1. Effect of neonatal $C5^-$ thymus graft on $C5^-$ mice. Adult $C5^-$ (B10.D20SN) hosts were thymectomized, grafted with neonatal thymus from $C5^-$ or $C5^+$ (B10.D2NSN) donors, irradiated, and reconstituted. Mice were immunized with C5/OVA/CFA followed by a booster immunization 2 wk later. Anti-C5 antibody was assayed by inhibition of C5-dependent hemolysis (6). The left panel represents the primary response (day 14) measured at a serum dilution of 1:25; the right panel represents the secondary response (day 21) measured at a serum dilution of 1:50. NSN, normal $C5^+$-immunized controls; OSN, normal $C5^-$-immunized controls; OSN Tx + OSN Thy, $C5^-$-thymectomized mice grafted with neonatal $C5^-$ thymus, irradiated and reconstituted as described; OSN Tx + NSN Thy, $C5^-$-thymectomized mice grafted with neonatal $C5^+$ thymus, irradiated and reconstituted as above. Shaded area represents the mean ± 2 SD of normal unimmunized $C5^-$ control sera.

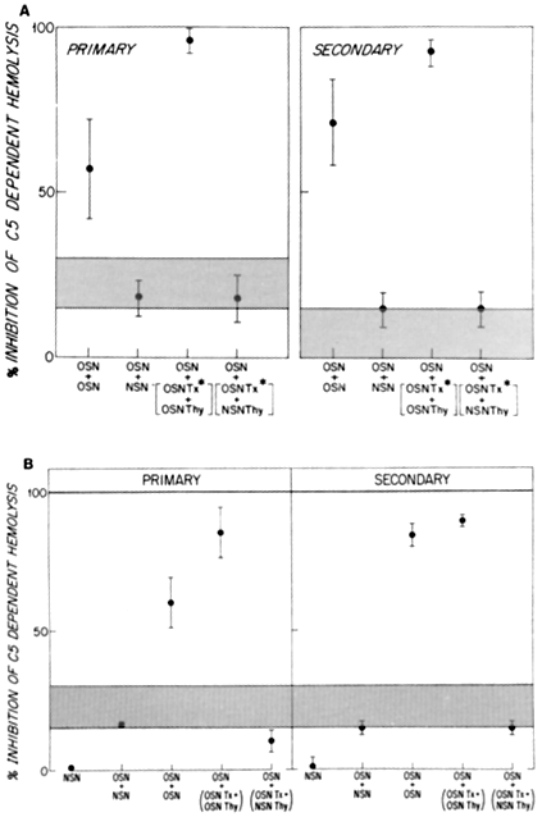

FIGURE 2. (*A*) Adoptive transfer of tolerance by immunized donors. NA spleen cells from both groups of immunize grafted mice described in Fig. 1 were injected into irradiated $C5^-$ recipients. NA spleen cells from unimmunized $C5^-$ and $C5^+$ donors into $C5^-$ hosts served as controls. All groups were immunized with C5/OVA/CFA on days 0 and 14 and anti-C5 response was measured by inhibition of C5-dependent hemolysis on days 14 and 21 at serum dilutions of 1:25 and 1:50, respectively. OSN + OSN, NA spleen cells from normal $C5^-$ donors into irradiated $C5^-$ hosts; OSN + NSN, NA spleen cells from normal $C5^+$ donors into irradiated $C5^-$ hosts; OSN + (OSN Tx* + OSN Thy), NA spleen cells from thymectomized $C5^-$ mice grafted with $C5^-$ neonatal thymus, irradiated and reconstituted, immunized with C5/CFA into irradiated $C5^-$ hosts. OSN + (OSN Tx* + NSN Thy), NA spleen cells from thymectomized $C5^-$ mice grafted with $C5^+$ neonatal thymus, irradiated and reconstituted, immunized with C5/CFA into irradiated $C5^-$ hosts. (*B*) Adoptive transfer of tolerance by unimmunized donors. Control and experiemental groups were as in *A*, except that NA spleen cells were transferred from unimmunized mice 3 mo after reconstitution. NSN, normal $C5^+$ controls; OSN + NSN, NA spleen cells from normal $C5^+$ donors into irradiated $C5^-$ host; OSN + OSN, NA spleen cells from normal $C5^-$ donors into irradiated $C5^-$ hosts; OSN + (OSN Tx + OSN Thy), NA spleen cells from thymectomized $C5^-$ mice grafted with neonatal $C5^-$ thymus, irradiated and reconstituted into irradiated $C5^-$ hosts; OSN + (OSN Tx + NSN Thy), NA spleen cells from thymectomized $C5^-$ mice grafted with neonatal $C5^+$ thymus, irradiated and reconstituted into irradiated $C5^-$ hosts.

TABLE I

Specificity of Tolerance Induction to C5 in Mice Immunized with C5 and OVA

Group	Anti-OVA
	OD
OSN Tx + OSN Thy	0.866
OSN Tx + NSN Thy	1.069
OSN + (OSN Tx + OSN Thy)	1.628
OSN + (OSN Tx + NSN Thy)	1.785

Anti-OVA antibody response measured by ELISA at sera dilution of 1:1,000. OD of unimmunized $C5^-$ and $C5^+$ mice was 0. OD, mean OD of individual mice in each group (six to eight mice/group) − (background + 2 SD). OSN Tx + OSN Thy, thymectomized $C5^-$ mice grafted with $C5^-$ neonatal thymus, irradiated and reconstituted with anti-Thy-1.2 + C-treated $C5^-$ bone marrow cells. OSN Tx + NSN Thy, thymectomized $C5^-$ mice grafted with $C5^+$ neonatal thymus, irradiated and reconstituted with anti-Thy-1.2 + C-treated $C5^-$ bone marrow cells. OSN + (OSN Tx + OSN Thy), nonadherent spleen cells of $C5^-$ mice grafted with $C5^-$ neonatal thymus into $C5^-$ host. OSN + (OSN Tx + NSN Thy), nonadherent spleen cells of $C5^-$ mice grafted with $C5^+$ neonatal thymus into $C5^-$ host.

To further establish that tolerance was induced in this system, we determined whether we could transfer the state of tolerance with lymphoid cells. NA spleen cells from both groups discussed above were adoptively transferred into irradiated $C5^-$ hosts (Fig. 2 *A*). The results show that $C5^-$ hosts that received cells from previously immunized $C5^-$ donors grafted with autologous thymus responded better than $C5^-$ controls in the 1° response. In contrast, both the $C5^-$ hosts receiving NA spleen cells from immunized $C5^-$ mice grafted with $C5^+$ thymus or from $C5^+$ controls failed to respond both in the primary and secondary response to C5. Because antigen in CFA has already been shown to maintain tolerance (6), we were concerned that successful adoptive transfer could be due in part to immunization of the first host. The experiment was therefore repeated by transferring cells from unimmunized $C5^-$ mice grafted either with $C5^+$ or $C5^-$ thymus. The interval between reconstitution of the primary host and adoptive transfer into the secondary host was extended to 3 mo. Again, serum C5 levels were undetectable in both groups. The results (Fig. 2 *B*) confirm the previous findings and clearly show that tolerance was transferred whether or not the donor was immunized with antigen in CFA. There is a striking difference in the response of $C5^-$ hosts repopulated with spleen cells of $C5^-$ mice grafted with $C5^-$ as opposed to $C5^+$ neonatal thymus in both the primary and secondary responses. $C5^-$ mice grafted with autologous young thymus responded better in the 1° response than intact controls.

To establish the specificity of tolerance induction, $C5^-$ mice with either $C5^-$ or $C5^+$ neonatal thymus grafts were immunized after reconstitution with both C5 and an irrelevant antigen, OVA in CFA. Mice tolerant to C5 responded to OVA as well as the nontolerant groups as measured by an ELISA (Table I). Furthermore, both groups of secondary hosts in the adoptive transfer experiments immunized with both antigens showed no difference in response to OVA. Thus, tolerance to C5 is antigen specific.

Discussion

The above results are, as far as we know, the first to demonstrate formally that tolerance to autologous protein antigen originates in the thymus. This is consistent with observations showing that the thymus is the site of tolerance induction to both allo and self MHC 2–5, 7), but inconsistent with the view that tolerance is induced at a prethymic stage (8, 9). Previous experiments have shown that T cells, but not B cells, are tolerant to C5 (6). In addition, both helper and suppressor T cells appear to be involved in the cellular mechanism (10). Since the antigen is required not only to induce but also to maintain tolerance to C5 (6), it seems paradoxical that unresponsiveness was induced in $C5^-$ mice engrafted with $C5^+$ thymus when the antigen C5 was undetectable in the serum. We have to assume that C5 known to be present on monocyte cell surfaces (11) was carried over by the thymus graft. Pro-C5 present inside the macrophage of $C5^-$ mice (12) is not tolergenic since $C5^-$ mice make anti-C5 antibody when immunized with C5 (6). Because the MHC of both $C5^-$ and $C5^+$ thymus grafts is the same, the only difference between the two groups of mice is the presence of the antigen.

Where and how then is tolerance to self antigen induced? Some controversy remains as to the role of epithelial cells of the cortex and the hematopoetic-derived macrophage/dendritic cells of the medulla and corticomedullary junction in the in-

duction of tolerance to alloantigen (2, 13). Our experiments did not distinguish as to which cells of the thymus graft were involved in the induction of tolerance to autologous soluble protein antigen. Nevertheless, it is reasonable to postulate that tolerance to C5 was induced in bone marrow-derived lymphoid cells of the $C5^-$ host by the presence of antigen on the radioresistant component of the $C5^+$ thymus graft.

Since helper T cells are required to make anti-C5 antibody (6), how are they rendered tolerant? Three cellular mechanisms should be considered: (*a*) clonal deletion, (*b*) immunoregulation by suppressor T cells; and (*c*) direct inactivation of the T cells by the antigen. Clonal deletion has been formally demonstrated for self MHC (5). Recently, this observation has been extended by two groups to non-MHC antigen (14, 15). There are also data consistent with clonal deletion as the mechanism for tolerance to class I alloantigens on cell surfaces (7). Whether clonal deletion is also applicable to autologous soluble protein antigen presented by class II MHC molecules is unknown. It is widely held that to induce T cell tolerance, antigen bound to self MHC must interact with a TCR. What is remarkable in the above model is that not only are the MHC loci of the $C5^-$ and $C5^+$ mice identical, but also that the TCR for C5 is presumably the same. Since it is now established that there is a single TCR for both antigen and self MHC, our results could support the hypothesis that antigen presentation by identical MHC to the same TCR is not necessarily different for induction of tolerance and immunity. Thus, it is unlikely that a hole in the T cell repertoire can explain tolerance to C5. $C5^+$ mice contain suppressor T cells that prevent production of humoral antibody to C5 (10) and the possibility that helper T cells are down-regulated by suppressor T cells must be considered. Since natural tolerance is so profound and longlasting, the latter could be only one fail safe mechanism to protect the host against autoimmunity. This phenomenon is therefore more likely explained by functional T cell inactivation by tolerogen without physical deletion as shown for both lysozyme (16) and cytochrome *c* (17). Further experiments with the C5 model will help to distinguish between T cell inactivation and deletion.

Finally, the finding that natural tolerance originates in the thymus may have clinical implications since thymectomy is known to result in autoimmune phenomena (18–20). If indeed a healthy thymus plays a role in protecting the host against development of autoimmune disease, transplantation of thymic tissue might be of benefit in its treatment.

Summary

C5-deficient mice grafted with thymus from C5-sufficient donors and immunized with C5 failed to make humoral antibody to C5, suggesting that the transfer of thymus had induced tolerance. Irradiated C5-deficient hosts repopulated with lymphoid cells from thymectomized C5-deficient mice grafted with C5-sufficient thymus also failed to respond to immunization with C5, thus showing that the state of tolerance can be adoptively transferred. These results demonstrate that natural tolerance to self-protein antigen is "learned" in the thymus.

We thank Deborah Wilkinson for her secretarial assistance.

Received for publication 5 July 1988 and in revised form 23 September 1988.

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