Testing Time-, Ignorance-, and Danger-Based Models of Tolerance

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In this study, we present data showing that tolerance to Ags in the periphery is not determined by the time at which the Ag appears, or by special properties of tissues in newborn mice or newly developing immune systems. We placed male grafts onto immunoincompetent female mice, allowed the grafts to heal for up to 5 mo, and then repopulated the recipients with fetal liver stem cells. We found that the newly arising T cells were neither tolerant nor ignorant of the grafts, but promptly rejected them, though they did not reject female grafts, nor show any signs of autoimmunity. We also found that the H-Y Ag was continuously cross-presented on host APCs, that this presentation was immunogenic, not tolerogenic, and that it depended on the continuous presence of the graft. In searching for the stimulus that might activate the host APCs, we analyzed mRNA expression with a highly sensitive real-time quantitative PCR assay. By using two different "housekeeping" molecules for comparison, we analyzed the message levels for several stress and/or inflammatory molecules in the healed grafts. We found that the long-healed grafts were not equivalent to "normal" skin because the healed grafts expressed lower levels of GAPDH. Altogether, these data suggest that acceptance vs rejection of peripheral tissues is not attributable to ignorance, timing-based tolerance, or special circulation properties of naive T cells in neonatal tissues. It is more likely attributable to an aspect of the context of Ag presentation that remains to be identified. ***The Journal of Immunology,*** **2001, 166: 3663–3671.**

This report addresses two currently held ideas. The first is the long-standing concept that there is an early period in ontogeny during which the developing immune system is particularly susceptible to tolerance induction, such that it becomes tolerant of Ags that are present at that time. The second is the newer conclusion that establishment of tolerance to skin cannot occur in adult mice because T cell trafficking into the skin occurs only during the neonatal period (1). We have tested both of these concepts and our data fit with neither.

Although evidence that newly developing immune systems are tolerance-prone dates back to the experiments in newborn mice from Medawar and colleagues (2), recent evidence revealed that these experiments may have been misinterpreted. Newborn mice are perfectly able to respond if given Ags in appropriate doses, with appropriate adjuvants, or on appropriate APCs (3–6). Furthermore, adult mice can be rendered tolerant if given large doses of Ag-bearing cells (4) or smaller doses of cells from which professional APCs have been removed (7, 57, 58). One interpretation from these results is that newborn and adult immune systems are both tolerizable and immunizable and that the decision to respond or become tolerant must therefore be governed by features other than age or timing of Ag expression (8). However, another interpretation, recently elaborated by Bretscher (9), is that the newborn is too old and has already passed through the tolerance-prone period. Therefore, tests for a tolerogenic period must be done earlier, at the inception of immune competence.

A number of experiments fitting these test criteria have shown that MHC or multiple minor histocompatability-mismatched grafts given before the development of immunocompetence can be rejected by a newly developing immune system (10–15). However, it could be reasonably argued that these experiments are not a valid test of time-based models. A number of models, including Bretscher's, are based on the hypothesis that antigenic exposure early in ontogeny is tolerogenic because of the low frequency of effector Th cells in the periphery at this time (9, 16–18). The number of Ags in MHC or multiple minor mismatched grafts could be greater than the number of tissue-specific self Ags to which the immune system must normally establish peripheral tolerance (19, 20) and consequently, the frequency of Th cells against these Ags would be high enough that any tolerance-prone period would be very short. Therefore, Bretscher suggested an experiment in which female recipients would be given APCs presenting the single minor Ag H-Y before the recipient has developed immunocompetence (9); Brestcher's prediction being that such early presentation of a single minor-histocompatability Ag would induce tolerance rather than the immunity that was seen when neonatal females were challenged with H-Y expressing APCs (4). Therefore, we grafted male and female skin to immunodeficient female recipients, allowed the grafts to heal for several months, then reconstituted the mice with fetal liver cells or a fetal thymus and followed the fate of the grafts.

Different models predict different outcomes of this experiment. Some time-based models, in which tolerance is based on the low frequency of helper T cells early in ontogeny, predict that the male skin, because it preexists, should be tolerated by the newly arising T cells (9, 16–18). Another recent time-based model, in which tolerance to skin is based on a particular property of neonatal skin that allows traffic of naive T cells, would predict that both the male graft and the normal adult female skin graft should be rejected (1).

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Ignorance models would predict that the male graft should be ignored until the animals are immunized (21). We found that the results fit with none of these predictions. The newly arising T cells rejected the male grafts while simultaneously becoming tolerant of peripheral self tissues. These data are strong evidence against time-based models and indicate that tolerance to skin Ags can occur readily without a period of T cell trafficking into neonatal tissues.

The Danger model would predict that recent male skin grafts would be rejected because of alarm signals sent by cells damaged during the surgical procedures, but that the long-healed skin should be accepted (22, 23). Thus, we were left with the question of what allowed the newly developing T cells to discriminate between the long-healed skin of the male donor and the normal peripheral tissues of the recipient, rejecting the former and becoming tolerant of the latter. In other studies of "well-healed" MHC or multiple minor mismatched grafts (10, 11, 15), the stimulus for rejection could have been cross-reactive environmental Ags (which may themselves be associated with danger signals) rather than the grafts themselves, making these studies difficult to interpret. To resolve these difficulties, Bingaman et al. (10) suggested that studies with H-Y may be enlightening. Though we used grafts differing only H-Y, an Ag that seems not to have any environmental mimics (7, 24), the healed grafts nevertheless were rejected. Thus, cross-reactive Ags were not the explanation for the rejection of healed male skin grafts. We also tested the distribution of the male Ag in the draining lymph nodes. Similar to studies with Ag expressed in normal kidney and islet tissue (25) we found that the Ags in the graft were continuously cross-presented in the draining lymph nodes, but unlike normal tissues, this cross-presentation was immunogenic. Therefore, we analyzed the long-healed grafts by immunohistology and quantitative real time PCR for molecules associated with inflammation. When compared with GAPDH, but not cyclophilin, the long-healed grafts appeared to have raised levels of several messages associated with stress, inflammation, and wound healing. These data indicate that some yet to be discovered aspect of the context of Ag presentation rather than the timing of Ag exposure is the critical factor in determining immunity vs tolerance.

Materials and Methods

Mice

Adult C57BL/6 (B6),[2] B6 nude, B6 nu/+, B6-MHC-II-knockout (KO), B10, B10.D2, normal and timed pregnant B10-RAG-2-KO (B10-RAG), and B6-TCRα-KO mice were obtained from Taconic Farms (Germantown, NY). C.B-17 SCID, as well as timed pregnant B6 and BALB/c mice, were obtained from the Frederick Animal Facility (National Cancer Institute, National Institutes of Health, Frederick, MD). All mice were housed in specific pathogen-free conditions, and B10-RAG mice received antibiotics in their drinking water (trimethoprim-sulfa). The National Institutes of Health is an American Association of Laboratory Animal Care-accredited facility.

Surgery, grafting, and reconstitution

Full-thickness skin grafts of ~2 cm in diameter were placed on the lateral thoracic wall of each mouse. They remained at ~1–2 cm in diameter after healing. In experiments in which mice were given two simultaneous skin grafts, the grafts were placed on either side of the body and were ~0.5–1.0 cm in diameter after healing. Grafts were considered rejected when at least 90% of graft tissue had disappeared or in the case of grafts undergoing "chronic" rejection, when the graft tissue became "shiny" subsequent to hair loss indicative of scar tissue. Adult syngeneic ovary grafts were placed in the ear pinnae by the same technique commonly used for cardiac grafts (26).

Thymectomy was performed by aspiration of both thymus lobes through a small incision in the skin just above the sternum and was confirmed by autopsy. Thymus grafting was performed by placing two to three lobes of fetal (day 15 or 18) or neonatal ($\leq$ 24 h old) thymus under the left kidney capsule.

Mice were immunologically reconstituted in the following four ways: 1) by grafting of neonatal thymuses to nude recipients; 2) by i.v. injection of 20×10^6 day-15 B6 or day-12 BALB/c fetal liver cells, respectively, to B10-RAG or C.B-17 SCID recipients; 3) by both thymus grafting and fetal liver cell injection of thymectomized B10-RAG recipients or 4) by i.v. injection of 50×10^6 spleen and lymph node (axillary, inguinal, cervical, popliteal, and mesenteric nodes) cells from unimmunized mice, and flow cytometry confirmed the presence of T cells.

Assay for Ag on APC in lymph nodes

Lymph nodes draining the skin grafts were made into a single cell suspension by squeezing the node apart between two layers of nylon mesh. The cells were irradiated (1500 rad) and cultured in titrated numbers with 20×10^3 Rachel cells in 0.2-ml round-bottom microwells. Rachel is a $CD4^+$ Th1 clone specific for H-Y/A^b generated from a (B6 $\times$ CBA/N) female mouse. Cultures were pulsed with [^{3}H]thymidine at 48 h and harvested ~15 h later to measure thymidine incorporation by liquid scintillation counting.

Detection of anti-graft CTL priming

To assess CTL priming in skin graft recipients and controls we gave the recipients i.v. 10^7 B cells from B6 male donors. The B cells were rigorously purified as described previously (7). The injection of purified B cells boosts the response of primed T cells but instead tolerizes naive T cells (7). CTL were assayed by the JAM Test (27). Briefy, 6×10^6 spleen cells from recipients (responders) were stimulated in vitro per 2-ml well (2 wells) for 6 days with 2×10^6 irradiated male spleen cells per well and then tested for ability to kill [^{3}H]thymidine-labeled male and female activated splenocyte Con A blasts at various responder to target ratios. The responder to target ratios are calculated from the number of responders originally cultured.

Histology

Heart, lung, kidney, liver, pancreas, small intestine, ovary, thyroid/parathyroid, and skin were fixed in 10% buffered formalin (Baxter) and embedded in paraffin. Serial sections were stained with hematoxylin and eosin by standard methods at Histo-Path of America (Millersville, MD).

Quantitative RT-PCR

Total RNA was extracted from the skin samples by using RNA STAT-60 (Tel-Test, Friendswood, TX). RNA was treated with 10 U of RQ1 DNase I (Promega, Madison, WI) for 30 min at 37°C. Samples were extracted with phenol/chlorofrom and RNA was precipitated with 0.3 M NaOAc and 2 vol of 100% ethanol. RNA was resuspended in diethyl pyrocarbonate-treated sterile water, and the RNA concentration was determined by measuring the OD_{260} nm. rTth DNA polymerase was then used to reverse transcribe and amplify 25–50 ng of total RNA in a single-tube assay with the Perkin-Elmer TaqMan EZ RT-PCR kit (Perkin-Elmer, Foster City, CA) with gene-specific sense and antisense primers and a probe fluorescently labeled at the 5′ end with 6-carboxy-fluorescein (28, 29). Primers and fluorescently labeled probes were then generated by using Primer Express software (Perkin-Elmer) and were synthesized by Perkin-Elmer. To avoid amplification of contaminating genomic DNA, primer pairs were selected that crossed intron/exon borders whenever possible. Samples were reverse transcribed for 30 min at 60°C and then subjected to 40 rounds of amplification for 15 s at 95°C and 1 min at 60°C with the ABI Prism 7700 sequence detection system as described by the manufacturer (Perkin-Elmer; Ref. 29). Sequence-specific amplification was detected as an increased fluorescent signal of 6-carboxy-fluorescein during the amplification cycle. Quantitation of gene-specific message levels was based on a comparison of the fluorescence intensity in the unknown mRNA sample to the fluorescence intensity from a standard curve of known mRNA levels. Amplification of the genes for mouse GAPDH or cyclophilin were performed on all samples tested to control for variations in amounts of RNA. Levels of gene-specific messages were graphed as normalized message units as determined from the standard curve. The data are expressed as a ratio of the transcript values obtained from long-standing skin grafts compared with their normal skin counterpart, or as TaqMan units that are relative values of transcript levels for a given gene, which have been normalized to a housekeeping gene (GAPDH). A no-template control was included in each amplification reaction to control for contaminating templates. For valid sample analysis, the fluorescence intensity in the no template control was required to be zero.

[2] Abbreviations used in this paper: B6, C57BL/6; KO, knockout; B10-RAG, B10-RAG-2-KO; DEC, dendritic epithelial T cell.

Results

Newly generated T cells reject healed male skin grafts

In our first series of experiments, we asked whether a healed graft carrying a single, weak, minor histocompatability Ag, would induce tolerance, be ignored, or induce immunity in T cell populations that were new thymic emigrants. We assessed the response of newly generated T cells by giving female B6 nude mice a nude male skin graft and 9 wk later, a female thymus. These thymuses were from normal B6 donors (Fig. 1*A*), or from B10-RAG donors (Fig. 1*B*) that have no functional T cells and must be repopulated by stem cells from the nude host before any T cell development can occur. The expectation was that newly maturing T cells from the female thymus should slowly seed the periphery in low numbers, encounter the H-Y Ag either in the male skin graft or in its draining lymph node (25, 30), and become tolerant. As a control to ensure that central thymic tolerance was functioning properly in these animals, we also grafted one cohort of nude females with a male B10-RAG thymus.

Fig. 1, *A* and *B,* shows that newly generated T cell populations rejected the long-standing male grafts, though the grafts had healed for 9 wk (or 14 wk, data not shown) and appeared as healthy as the host's own skin. The rejection did not require the transfer of mature T cells with the thymus graft because the B10-RAG thymus grafts, like the normal B6 thymuses, lead to rejection. Recipients reconstituted with a male thymus did not reject the male skin (Fig. 1*B*) showing that central tolerance mechanisms were functionally intact and able to induce tolerance in the newly developing T cells, but peripheral tolerance to the grafts did not occur. Thus it appeared that mature naive T cells and, more surprisingly, newly developing T cells, were immunized rather than tolerized by the peripheral Ag on the healed grafts, despite the low frequency of responding cells to H-Y and the lack of cross-reactive environmental Ags to drive the response (7, 24).

In the experiments above, the bone-marrow-derived precursors were from adult marrow, and the thymuses in which they developed were from neonatal animals. It remained possible that peripheral tolerance depends on some property intrinsic to fetal but not adult precursor cells or thymuses. It has also been reported that nude mice have a small number of mature T cells, which may have contributed to the rejection process. Therefore, we reperformed the grafting experiments in B10-RAG recipients by reconstituting with male or female fetal liver cells. In addition, to test whether the maturity of the thymus itself has an influence, we thymectomized a cohort of female B10-RAG mice and reconstituted them with a day-15 fetal B6 thymus plus an injection of day-15 fetal liver cells (Fig. 1, *C* and *D*). Once again, in both groups, we found that the female mice reconstituted with female fetal tissues rejected the long-standing male grafts. Thus, T cells generated from fetal precursors, in a fetal thymus, in hosts that (unlike nudes) have no preexisting T cells, went on to reject a healed preexisting male skin graft, a surprising result that was not predicted by any current model of immunological tolerance. The mice did not reject grafts of female skin, showing that some aspects of peripheral tolerance were intact, and those given male thymuses did not reject male skin, showing that central tolerance was also intact. Rejection of preexisting grafts was not peculiar to B6 or B10 mice, as C.B-17 SCID mice reconstituted with day-12 BALB/c fetal liver cells also rejected a preexisting B10.D2 minor-histocompatibility different skin graft (not shown and Ref. 59).

Lack of autoimmune responses in reconstituted mice

The finding that the long-standing male grafts were rejected by newly generated recent thymic emigrants counter the expectations of most time-based models because these models hold that a preexisting Ag should induce tolerance. They counter the ignorance model because the graft Ags are peripheral Ags that should be ignored. They counter the Danger model because the grafts had healed. Healthy tissues should not immunize.

However, one recently elaborated time-based model did offer a possible explanation for the rejection. This model suggests that tolerance to peripheral Ags cannot be established in adult mice. It was proposed that establishment of tolerance to skin, for example, requires that naive T cells traffic into the skin, which occurs only in the neonatal period (1). If this were the case, we would expect to find general signs of peripheral autoimmunity, as has sometimes

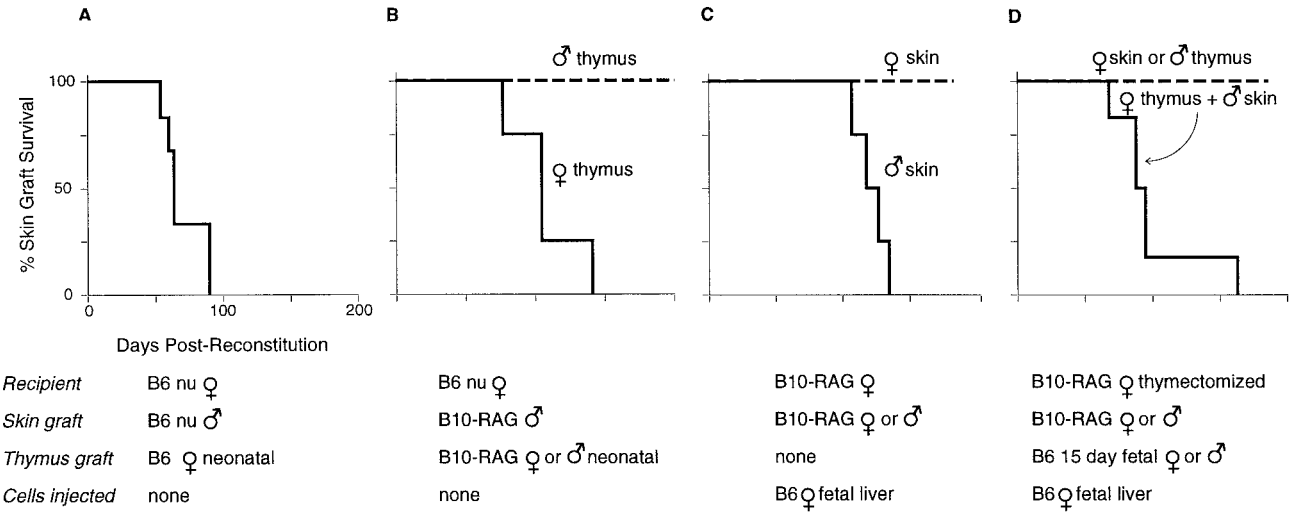

FIGURE 1. Well-healed skin grafts induce immunity. *A,* B6 nude female mice ($n = 6$) were given a B6 nude male skin graft and 9 wk later were given a neonatal B6 female thymus graft. *B,* B6 nude female recipients received a B10-RAG male skin graft and, 9 wk later, a neonatal male (dashed line; $n = 4$) or female (solid line; $n = 4$) B10-RAG thymus. *C,* B10-RAG female recipients ($n = 4$) received two skin grafts, a B10-RAG female (dashed line) and male skin graft (solid line). Eleven weeks later, they were reconstituted with day-15 B6 female fetal liver cells. *D,* Thymectomized B10-RAG female recipients received a B10-RAG male skin graft and 7 wk later were reconstituted with day-15 B6 female fetal liver cells and thymus graft (solid line; $n = 6$). Controls (dashed line) were similarly treated except they received a male fetal thymus graft ($n = 1$) or a female skin graft ($n = 2$).

been seen in other systems (31, 32). In addition, because we used adult mice, there is no possibility for traffic of T cells into neonatal skin for the establishment of tolerance. Thus, we would expect to see either heavy lymphocytic infiltration or rejection of the syngeneic skin grafts based on the data/conclusions reported by Alferink and colleagues in their transgenic system (1). Although the acceptance of female skin grafts, the long life span of the mice after reconstitution, and an absence of splenomegaly or lymphadenopathy (not shown) suggested that the mice were not autoimmune, we nevertheless took a further look at a potential generalized lack of self-tolerance. Nine to 21 mo after reconstitution, we tested nine different organs for signs of autoimmunity such as lymphocytic infiltration and/or tissue destruction (14 reconstituted and 6 age-matched normal mice were analyzed). Ovaries, thyroid/parathyroids, and skin appeared normal in all mice and there was no extensive lymphocytic infiltration of parenchymal tissue or damage to tissues that are highly sensitive to autoimmune destruction, such as glomeruli and pancreatic islets (Fig. 2, *A–D*). We did find some weak lymphocytic infiltration of the intestinal lamina propria in a few mice, and some focal perivascular nodules of lymphocytic infiltration in one or more organs, including the kidney, pancreas, heart, lung, and liver of all mice. However, these types of focal infiltrates were also found in all similarly aged normal mice.

Although all of the peripheral tissues appeared healthy, it was possible that the adult reconstituted mice were not truly tolerant of self-tissues but instead ignorant of them (1, 21). For example, the lack of rejection of female skin grafts did not necessarily mean the mice were tolerant of female skin. The long-standing female skin grafts could have stimulated a primary response that was simply too weak to cause rejection. Therefore, we transplanted mice that had rejected their preexisting male but not female skin graft with a second syngeneic female skin graft to boost the response. None of these mice rejected the second (or the first) female graft (Fig. 2*E*) and no infiltration of the graft was evident on histological

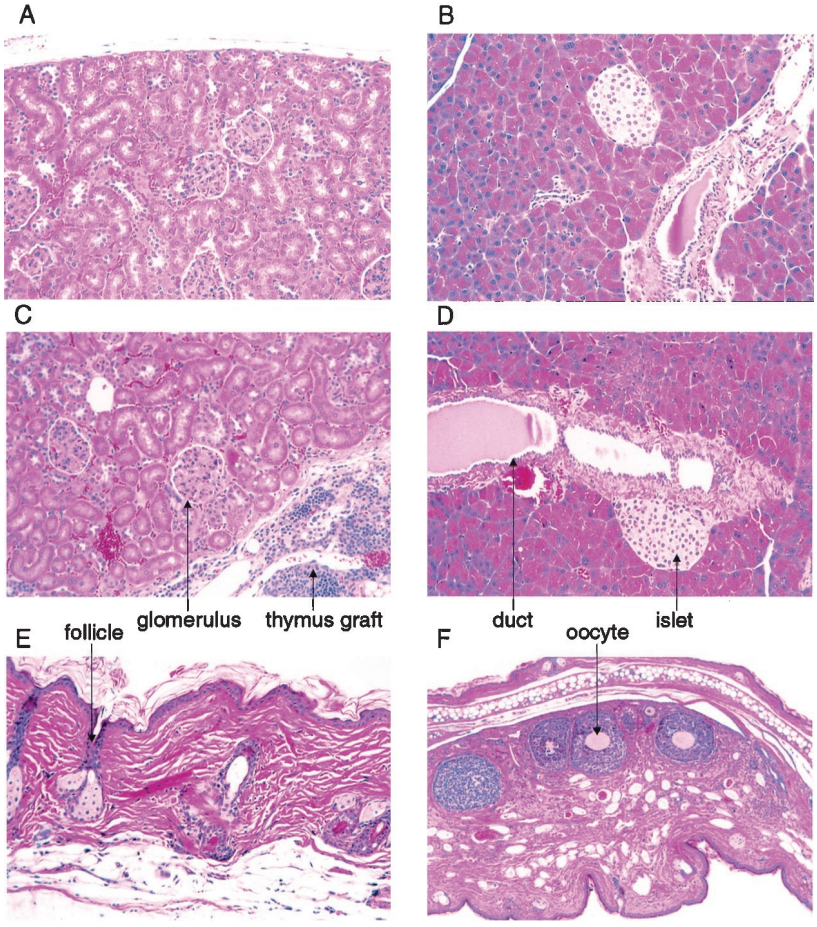

FIGURE 2. Lack of spontaneous or graft-induced autoimmune destruction in reconstituted mice. *A* and *B*, Representative hematoxylin- and eosin-stained tissue sections of kidney and pancreas from reconstituted mice taken ~1 year after they had rejected a preexisting skin graft. Nine different tissues (see *Materials and Methods*) were histologically analyzed from 14 reconstituted immunodeficient mice (including B6 nudes with thymus grafts and B10-RAG-KO and C. B17SCID mice given fetal liver) sacrificed 9–21 mo after reconstitution, as well as from six normal B6 mice at 8–22 mo of age. Kidney (*A*) and pancreas (*B*) (magnification ×200) taken from a 20-mo-old ungrafted control C57BL/6 mouse. *C*, Kidney (magnification ×200) taken 21 mo after thymus grafting from a B6 nude female that had rejected a 9-wk standing male graft. The reconstituting thymus is visible in the lower right corner. No glomerulitis is present. *D*, Pancreas (magnification ×200) taken 14 months after reconstitution with day-12 BALB/c fetal liver cells from a C.B-17 SCID that had rejected a 19-wk standing B10.D2 skin graft. The pancreatic islets are normal. *E*, Female B10-RAG skin graft (magnification ×200) 13 wk after grafting. Mice that rejected their male but not female skin graft in Fig. 2*C* were given a second female skin graft ($n = 4$). None of these mice rejected the graft. The graft shown is from a B10-RAG female that had been grafted with both male and female B10-RAG skin and 11 wk later reconstituted with female B6 fetal liver cells. This female rejected the standing male graft 18 wk after reconstitution, but accepted the female graft. She was re-grafted with female skin 26 wk after reconstitution and histology was performed on the second graft 13 wk later. *F*, Syngeneic ovary graft (magnification ×100) in ear pinna, 10 wk after grafting. Tissue was taken from a B6 nude female that was given a B6 female thymus graft and at the same time had one of her ovaries removed and placed in the ear pinna. No folliculitis is present and oocytes exist in varying stages of development.

examination, which strongly suggests that these mice were solidly tolerant of their own (female) adult skin, though they had rejected the male skin.

The simplest explanation for our results would be that tolerance to skin Ags is determined centrally. Although several Ags (e.g., insulin, myelin basic protein, and ocular S-Ag), the expression of which was thought to be restricted to a peripheral tissue, were found on further examination to be expressed at low levels in the thymus (33–35), there is some evidence for a skin Ag that is not on thymic epithelium- (36) or bone marrow-derived cells (37, 38). Thus, in the face of such potential skin-specific Ags, the newly arising T cells in adult mice appeared to become tolerant of female skin, but rejected the male skin.

To test for self tolerance to a second tissue, we challenged a set of B6 nude female mice that had been reconstituted with a B6 neonatal female thymus by grafting with a syngeneic ovary. These mice showed the same strong tolerance for a peripheral organ, as there were no signs of infiltration or damage to the graft (Fig. 2*F*) or to host ovaries. Taken together, these data establish that peripheral tolerance does not require a neonatal tolerance window. Tolerance was established despite the fact that the newly arising T cells developed in an adult body. The only reactivity we saw was to the long-standing, male skin graft.

Constitutive presentation of skin graft Ag in draining lymph nodes by host APC

"Ignorance" models postulate that peripheral tissue Ags go undetected by T cells because APCs do not capture them and they consequently do not make it into lymphoid tissue (Ags in lymphoid tissue being immunogenic). However, our healed grafts did not go undetected but were rejected. To test whether the graft Ags were carried into lymphoid tissue, perhaps by a mechanism unanticipated by ignorance models, we used a sensitive in vitro test for the presence of H-Y. Five months after grafting male skin onto immunoincompetent hosts, we tested the cells from nodes draining the skin grafts as APCs in titrated in vitro tests, asking whether Rachel, a $CD4^+$ T cell clone specific for H-Y/I-A^b, would respond. Further, to test whether such presentation was attributable to the migration of any remaining donor APC or whether host APCs were mostly involved, we also tested the APCs from nodes draining an MHC class II-negative skin graft. We found that the graft Ag was indeed presented in the draining nodes and that host, rather than donor, APCs accounted for most of this presentation. Fig. 3 shows that Rachel responded well to lymph node cells draining a male graft, whether or not the graft expressed MHC class II, but not to lymph node cells from ungrafted female control mice (Fig. 3, *A* and *B*). Thus, host APCs are able to present graft Ags as long

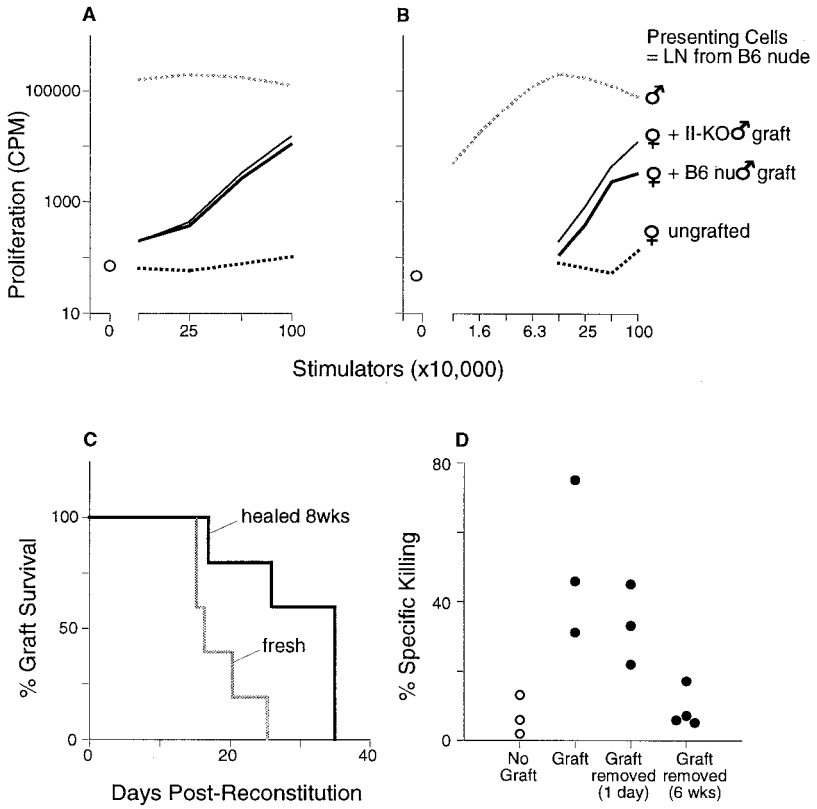

FIGURE 3. Ag of a long-standing skin graft is constitutively presented by host APC in the draining lymph nodes. *A* and *B*, B6 female nude mice received a B6 nude male (thick black line) or B6-MHCII-KO male (thin black line) skin graft or no graft. Fifteen to 20 wk later, axillary lymph nodes draining the skin graft were harvested, irradiated, and used as stimulators for Rachel, a $CD4^+$ anti-H-Y T cell clone. Lymph node cells from a B6 nude male were used as a positive control (dotted gray line), and from ungrafted B6 nude females as a negative control (dotted black line). *A* and *B* depict two different experiments. *C*, B6 female nude mice received a B6-MHCII-KO male skin graft 8 wk before (black line; $n = 5$) or 1 day after (gray line; $n = 5$) reconstitution with naive B6 female spleen and lymph node cells. *D*, B6 nude female mice received a B6 nude male skin graft, or no graft, and the graft was allowed to heal. Nine weeks after grafting, all mice received B6 nu/+ female lymphocytes i.v. In one cohort of mice the skin grafts were removed and replaced with B6 nu/+ female skin 1 day before lymphocyte injection and in another cohort the grafts were replaced 6 wk before lymphocyte injection. Two weeks after injection of female lymphocytes, all recipients were challenged with 10^7 purified B cells from B6 male mice, and 3 wk later, their spleen cells were cultured with male stimulators to test for CTL priming. Specific killing of male targets (killing on female targets has been subtracted) is depicted.

as 5 mo after grafting. By comparison with titrated male lymph node cells, where every APC is expected to express H-Y, the APCs in nodes draining the male skin grafts were ~60 fold less efficient, cell for cell. If we assume that the non-APCs in the nodes have little positive or negative effect in our assay, this suggests that about one in every 60 host APCs presented enough H-Y Ag to stimulate the T cell clone.

Because the process of preparing the lymph node APCs for the in vitro test induces activation and up-regulation of costimulating molecules (39, 40), their ability to stimulate a T cell clone in vitro does not necessarily reflect their stimulatory capacity in vivo. It merely indicates that the host APCs have captured the donor skin Ag and carried it to the node. In vivo, the APCs might have remained quiet, presenting their captured Ag in a tolerogenic fashion, in a manner similar to the APCs from the nodes draining islets and kidneys in transgenic mice expressing OVA under control of the rat insulin promoter (25). However, Fig. 3*C* shows that this was not the case. The cross-presentation by host APC was immunogenic not only in vitro, but also in vivo, as even well-healed, MHC-II-negative male skin grafts, where only host APCs can present to Th cells, were rejected on reconstitution with naive female T cells. Thus, the H-Y Ag was present in the draining nodes, it was present on host APCs, and it was immunostimulatory. The rate of rejection of healed grafts was significantly slower than that of the fresh grafts ($p = 0.03$, logrank analysis), which may indicate that the fresh surgical damage and consequent tissue necrosis (40) provided a greater stimulus for APC activation and rejection.

It has been demonstrated that dendritic cells in nodes can cross-present Ags captured from other dendritic cells. Because the lifespan of these cells is not known, there was a remote possibility that the immunogenic H-Y Ag in the draining nodes had been captured from dendritic cells migrating from the grafts early after grafting and that this Ag was still being presented 5 mo later. To test whether the presence of the Ag in the nodes required the continued presence of the graft, we gave nude female mice a male skin graft and later removed the male graft (and replaced it with female skin), or not, and then injected female lymphocytes to see whether they could become primed to the graft Ag in the absence of the graft. Fig. 3*D* shows that female T cells were primed to make anti-H-Y CTL when the graft was left on or when it was removed 1 day before injection of the female lymphocytes. However, they were not primed if the graft was removed 6 wk before female lymphocyte injection, indicating that the functional lifespan of activated APC in the draining lymph node is relatively short. Similar results were obtained in RAG-KO female hosts when a RAG-KO male skin graft was removed before T cell injection (not shown and Ref. 59). Thus, it appeared that host APCs continue to capture and present graft Ags on both MHC class I and II for months after transplantation.

Long-standing skin grafts are not completely normal

Though the constitutive cross-presentation shown here is not predicted by the ingnorance model, it is hardly surprising, as Kurts and colleagues have shown a similar phenomenon for OVA expressed transgenically on islets and kidney cells (25). However, the transgenically expressed Ag in their study led to a cross-presentation that was tolerogenic, whereas constitutive cross-presentation from a long-established graft in our study was instead immunogenic. One possibility for the difference comes from the danger model, which postulates that signals from stressed cells should activate local APCs, such that they should present captured Ags along with costimulatory signals to passing T cells (23, 40–42). This impelled us to have another look at the assumptions under which we were operating. Up to this point, we had assumed that a long-standing graft is as healthy as normal tissue. For time-based models or ignorance models, the health of a grafted tissue is not terribly relevant to whether it can induce tolerance. However, to some of the context-based models, the health of the graft is crucial. Therefore, we decided to have a closer look to determine whether the skin grafts were indeed as quiescent as normal skin or whether there may be some differences between the graft and normal skin that could explain rejection.

To minimize any potential positive or negative effects attributable to an adaptive immune response, we took samples from graft recipients that had not been reconstituted with T cells, thus ensuring that there was no interference by T cells capable of responding to the graft. Furthermore, because normal mouse skin contains $\gamma\delta$TCR dendritic epithelial T cell (DEC) populations that may contribute to the normal healing process by producing epithelial cell growth factor (43), we used TCRα-KO female recipients and male donors, which lack mainstream TCR$\alpha\beta$ T cells but contain the $\gamma\delta$DEC cells and therefore should heal normally. Fig. 4 shows that after reconstitution with naive female lymphocytes, all of the grafts were rejected, illustrating that long-healed male skin is rejected even in the presence of the DECs.

When we analyzed the state of health of these grafts, healed onto unreconstituted recipients, by standard histology and by immunochemical staining for Keratin-6 (not shown), a protein the expression of which is increased after wounding (44), we found that the long-standing grafted skin appeared similar to normal skin with the exception that there were reduced numbers of glands in the grafted skin. Similar to the data of Bingaman et al. (10), we found the levels of RNA for several proinflammatory cytokines (IL-1β, IL-6, TNF-α, GM-CSF) were similar between normal and long-standing grafted skin when analyzed by conventional (nonquantitative) PCR. However, a more sensitive method may be necessary to detect relevant differences between grafted and normal skin. When we used real-time quantitative PCR to look at the mRNA levels of several molecules known to be expressed in damaged or recovering cells, or known to be involved in inflammation and immunity, we initially saw some striking differences.

Normalizing mRNA levels to GAPDH, we compared long-healed grafts (4 mo after transplantation) with freshly grafted skin (4 days after transplantation, a positive control for surgery-induced damage and inflammation); and with normal skin from the grafted recipients. Fig. 5*A* shows that long-healed grafts had higher levels than normal skin for 6 of the 10 messages. To verify these increases in stress/inflammation molecules, we analyzed more healed grafts and normalized the mRNA in relation to both

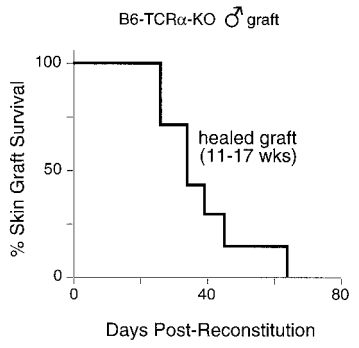

FIGURE 4. The presence of dendritic epidermal T cells does not allow tolerance to long-standing grafts. B6-TCRα-KO female mice were given a B6-TCRα-KO male skin graft and 11 or 17 wk later were reconstituted by injection of B6 female spleen and lymph node cells ($n = 7$).

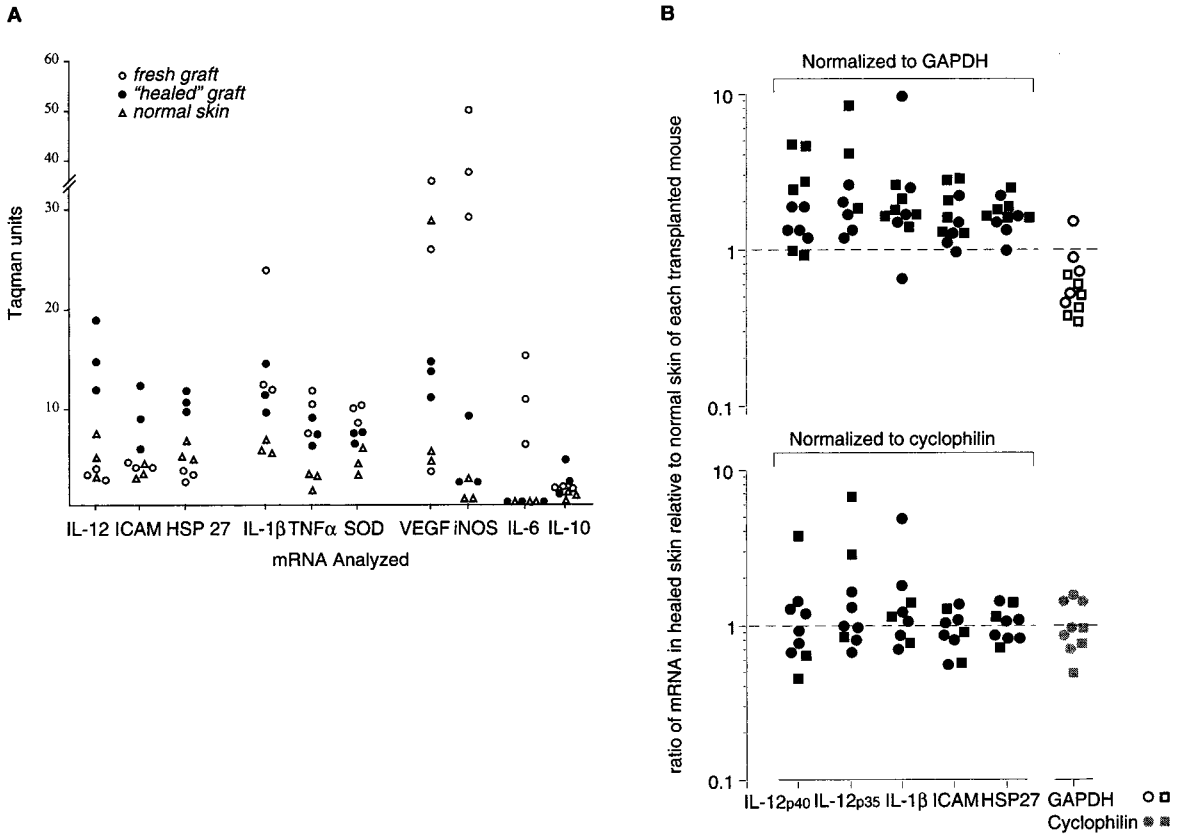

FIGURE 5. Altered mRNA expression in long-standing skin grafts. *A*, Female B6-TCRα-KO mice were given male or female B6-TCRα-KO skin grafts and 16 wk (●; male grafts) or 4 days (○; female grafts) later the grafts were removed and RNA extracted for real-time quantitative RT-PCR analysis of the indicated mRNA by using the Perkin-Elmer TaqMan EZ RT-PCR kit and normalization of mRNA amounts based on GAPDH expression (see *Materials and Methods*). For comparison, normal host skin, adjacent to the graft was also analyzed (△). Results for individual mice are depicted and are expressed in message units as determined from comparison with standard curves specific for each gene. Because different genes had different scales of amplitude, due to the specific efficiency of their primers and probes, we multiplied or divided their values by 10 so that the results of the normal skin samples were around 1 to be able to present them in the same figure. *B*, Female immunodeficient recipient mice (B6-TCRα-KO or B10-RAG) were given skin grafts (B6-TCRα-KO or B10-RAG grafts) from male (squares, $n = 3$) or female (circles, $n = 6$) donors, and 2–7 mo later, the grafts were removed and RNA extracted for real time quantitative RT-PCR analysis as in *A*. Normal skin adjacent to the graft from each mouse was also analyzed and the results are depicted as the fold difference between graft skin and the corresponding normal skin for each mouse (dashed line at a value of 1 indicates where the graft and normal skin have the same level of mRNA expression). In the *top panel* the values were normalized based on the level of GAPDH (■, ●) expression (this panel also includes values for the three mice analyzed in *A*), and in the bottom panel, cyclophilin (■, ●) expression was used for normalization. The raw values (nonnormalized) for GAPDH (*top*, □, ○) and cyclophilin (*bottom*, gray) are also shown.

GAPDH and cyclophilin expression. Fig. 5*B* shows that when normalized to GAPDH, the increases in mRNA for stress/inflammation molecules were very reproducible. However, these increases were far less apparent when the mRNA was normalized to cyclophilin expression. The level of cyclophilin mRNA expression was approximately the same in normal skin and the long-standing graft, whereas GAPDH was generally lower in the long-standing graft. Thus, there appears to be a decrease in GAPDH expression in the healed skin grafts when compared with normal skin.

Discussion

In summary, by using sensitive in vivo and in vitro techniques, we found that skin grafts are not equivalent to normal skin even many months after grafting, that they remain continuously immunogenic through cross-presentation on host APC, and that they are rejected by newly arising T cells that nevertheless become tolerant of their own tissues.

These are not the first results to challenge the time-based models of tolerance. For example, Lambert's group showed that neonatally tolerant mice are tolerant at the level of CTL but not Th cells (45), Coutinho's group showed that they contain large numbers of polyclonally activated cells (46), and Mahana et al. (3) found that normal neonates are able to make Abs against self Ags. Furthermore, it was recently shown that neonates can make normal CTL and Th responses if given the appropriate Ag doses, adjuvants, or APCs (4–6). In response to these results, proponents of time-based models have argued that neonates are not appropriate test animals because the tolerogenic period is already over in neonatal animals, and that the Ag must therefore be introduced even earlier if it is to be obligatorily tolerogenic (9, 17, 18). However, this view also has been challenged in several ways. As early as 1979, it was shown that skin grafts that had been grafted onto nude mice before reconstituting the recipients with a thymus graft were rejected by the newly generated T cells (11), and similar results were later obtained in SCID and RAG-KO mice (10, 15). Furthermore, grafts given to chicken or sheep embryos before the development of immunocompetence also were rejected when the animals developed immune competence (12–14). However, in all of these studies, the grafts differed from the hosts at MHC and/or multiple minor loci, and therefore the frequency of anti-graft T cells was most likely greater than the frequency of T cells to any peripheral tissue-specific Ag. Therefore, it could be argued that these studies were not

valid tests of those time-based models in which tolerance is thought to occur as a result of a low frequency of Th cells.

H-Y is a single, weak minor histocompatibility Ag. In unprimed mice, the frequency of responding T cells is undetectably low. It lacks environmental mimics (7, 24), and the response requires cellular collaboration between T cells (47–49, 60). In these ways, it is quite likely to mimic a conventional peripheral self-Ag, and for these reasons, both Bretscher (9) and Bingaman et al. (10) suggested that the ultimate test of peripheral tolerance models should be done with H-Y. Nonetheless, by using this antigenic system, we found that both mature naive, and, most surprisingly, newly arising T cells, rejected the long-standing male skin grafts, while at the same time, these T cells became tolerant of their own tissues.

None of the current time-based models are easily able to incorporate our data. Most time-based models would have predicted that newly arising T cells would have accepted the long-standing male skin grafts. Some of the models (9, 16, 17) might argue that the male grafts were too small to serve as an adequate source of Ag during the early tolerizable-only period in which the frequency of T cells is low. Though we cannot completely rule this out, it seems very unlikely, given that graft Ags were readily detected in the draining lymph nodes and the male grafts were quite large (up to 2 cm in diameter) and that other, much smaller organs, such as ovaries and islets, were tolerated. A more recent time-based model, which suggests that establishment of tolerance to skin occurs only in the neonate because neonatal skin allows traffic of T cells whereas adult skin does not (1) would have suggested that the newly arising T cells in an adult body would have rejected both the male and female adult grafts. Our data show, to the contrary, that tolerance to skin can be established to adult skin in an adult animal and does not require any property unique to neonatal tissues.

Our data are also difficult for the ignorance model of Zinkernagel (21), which would have predicted that the newly arising T cells would have "ignored" both the male and female grafts. This model proposes that peripheral Ags are not captured by host APCs and presented on MHC class I molecules unless the Ag is directly inserted into an APC, for example, by virus infection. Clearly, however, like many other minors that have been shown previously to be cross-presented in vivo (50, 51), the H-Y Ag reached the draining nodes, was presented by host APCs, and was able to activate CTL there. Thus, the ignorance model will need to be provided with additional or amended assumptions to explain these data.

Although apparently ruling out existing time-based models, our data also do not fit easily with the Danger model, which suggests that tolerance will be established to an Ag unless APC costimulation is induced by signals from stressed or damaged tissues (22, 23). Though the longstanding grafts were grossly normal macroscopically, histologically, and by conventional PCR (Ref. 10 and our unpublished data), when analyzed with a sensitive quantitative PCR assay, the level of expression of GAPDH was decreased in comparison with normal skin. It remains possible that abnormal expression of other molecules not yet examined may provide a better explanation for the immunogenic cross-presentation leading to graft rejection that we have observed. Rejection was not the result of long-lived APC that were stimulated to cross-present graft Ags at the time of grafting. Instead, we found that host APCs had a short functional lifespan (<6 wk), but the graft Ags were continuously presented in the draining lymph nodes for at least 5 mo after grafting. Thus, there is likely to be continuous migration of activated APCs that have captured skin graft Ags and, unlike APCs migrating from normal internal tissues such as kidney and pancreas (25, 52), these APCs are immunogenic.

Finally, the finding that the female graft was accepted whereas the male was rejected although both tissues potentially continue to generate activated APCs, suggests that tolerance can be established in the face of costimulation. The most likely explanation for this paradox lies in the size (antigenic load) of the two different tissues. Consistent with this view, it has been shown that increasing the size of a skin graft decreases the likelihood of rejection (53, 54). In our experiments, the H-Y Ag expression is restricted to the male skin graft while any skin-specific Ags on the syngeneic female graft are present not only on the graft but also on the rest of the host skin, a very large tissue. Should autoreactive T cells be activated by the syngeneic graft or by a few activated APC draining normal skin tissue, they would kill a few target tissue cells, as well as the activated APCs that stimulated them. However, because CTL killing induces apoptosis and such apoptotic death does not activate resting APCs, the self Ags in the dying tissues would be presented by nonactivated APCs (40, 55, 56), which would be tolerogenic. Therefore, the response would end and tolerance would eventually result. This would not occur for the male graft because the tissue expressing the Ag is much smaller, allowing the graft to be eliminated before the Ag presentation becomes tolerogenic.

Altogether, these studies demonstrate that it is not the timing of Ag exposure that determines the induction of tolerance vs reactivity. We are left with the context of Ag presentation as the determining factor. Dissecting the difference between tolerogenic and immunogenic cross-presentation by APC in lymphoid tissue is a critical next question. By characterizing the differences, we may find ways to manipulate Ag presentation to produce tolerance vs immunity at our discretion.

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