The fetus represents a foreign entity to the maternal immune system, yet this “natural” allograft is not normally rejected. This unique situation provides a physiologic system to evaluate peripheral tolerance in which the maternal immune system is challenged with relatively rare Ags not previously encountered in the thymus. Using H-Y-specific TCR transgenic mice, we demonstrate that T cells specific for fetal Ags decrease in an Ag-specific manner during pregnancy and remain low postpartum, the result of an encounter with fetal cells expressing the appropriate MHC/peptide complexes. The finding that placent al trophoblasts can induce Fas-mediated death of T cells is consistent with peripheral clonal deletion as one mechanism of tolerance. The remaining clonotypic T cells are unresponsive to antigenic stimulation, although neither TCR nor coreceptor is down-regulated. Our study demonstrates that recognition of fetal allogeneic Ags by maternal T cells results in tolerance induction of reactive T cells via multiple mechanisms. The Journal of Immunology, 1998, 160: 3086–3090.

Materials and Methods
Mice
αH-Y TCRtg mice bearing TCRs specific for H-Y/Dβ were bred to mice in which the recombinase activating gene-2 (RAG-2)1 had been eliminated by homologous recombination (RAG−/−) as previously described (14). Timed pregnant RAG−/− H-2b or H-2d TCRtg mice were generated by mating females for 18 h with C57BL/6 or BALB/c males (National Cancer Institute, Frederick, MD), respectively. The day that the pair was separated was counted as day 1 of gestation. In some experiments, RAG+/− H-2b or RAG+/− H-2d TCR were used. Pregnant females were sacrificed at day 7, 14, or 18 of gestation or allowed to give birth and then sacrificed 5 or 21 days postpartum.

Breeding pairs of C57BL/6J (B6), B6Smn, C3H-gld (B6αβ), and C3H/HeJ-gld (C3Hβγ) were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our facility. To isolate trophoblasts, timed pregnant mice were generated by mating B6αβ females with either B6αβ × C3HβγF1 (gld/gld) or (B6 × C3Hβγ)F1 (wt/gld) males for 12 h. The day that the pairs were separated was considered day 1 of gestation.

Flow cytometry and reagents
Spleen and pooled lymph nodes (mesenteric, axillary, and inguinal) were harvested from each animal and single-cell suspensions prepared and counted. Cells (1 × 10⁶) were stained with anti-CD4, anti-CD8, anti-Thyl.2, and anti-IgM (PharMingen, San Diego, CA) or anti-clonotypic TCR (T370 (15), generous gift of Dr. Elizabeth Shores, Center for Biologies Evaluation Research and Review, Bethesda, MD) and analyzed on a

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Abbreviations used in this paper: RAG-2, recombinase activating gene-2; Fas-1, Fas-ligand.
FIGURE 1. Specific elimination of H-Y-reactive T cells in peripheral lymphoid tissues. H-2^d, oH-Y TCRtg RAG^+/- females were mated with C57BL/6 males, then analyzed at various time points during pregnancy and postpartum for changes in cell number and, by flow cytometry, for expression of the clonotypic TCR (recognized by the mAb T370), CD8, and Thy-1 in the spleen (A) and the lymph nodes (B): nonpregnant (day 0), n = 20; day 7, n = 4; day 14, n = 13; day 18, n = 4; day 21 postpartum, n = 5; and day 21 postpartum, n = 4. C, H-2^d or H-2^b TCR transgenic RAG^+/- females were mated with C57BL/6 or BALB/c males, respectively, and analyzed by flow cytometry for the presence of H-Y-specific T cells at day 14 of gestation (H-2^b, n = 13; H-2^d, n = 5) and 5 days postpartum (H-2^b, n = 5; H-2^d, n = 6), then compared with nonpregnant controls (H-2^b, n = 20; H-2^d, n = 7).

The number of clonotype-expressing T cells has been normalized to H-2-matched nonpregnant controls for comparison. (Clonotypic T cell recovery from H-2^b spleens: nonpregnant, 1.8 ± 0.4 × 10^6; day 14, 4.1 ± 1.5 × 10^5; 5 days postpartum, 2.5 ± 0.6 × 10^6; and from H-2^d spleens: nonpregnant, 16.8 ± 2.9 × 10^5; day 14, 14.8 ± 2.1 × 10^6; 5 days postpartum, 5.8 ± 0.6 × 10^7). D, Nonpregnant H-2^b TCR transgenic RAG^+/+ (n = 3) or day 14 pregnant females mated to C57BL/6 males (n = 6) were sacrificed, and lymph node and spleen cells were counted and then analyzed by flow cytometry for expression of CD4, CD8, and T370.

FACSScan cytometer (Becton Dickinson, Mountain View, CA) after gating on viable lymphocytes. Cell recovery of T cell subsets was determined by multiplying the total cell recovery from that tissue by the percentage of that subset present in the tissue as determined by flow cytometry.

Isolation of trophoblasts

Isolation of trophoblasts was performed as previously described (16). Briefly, placenta from 18-day pregnant mice were harvested aseptically, digested with 0.5% dispase (Boehringer Mannheim, Indianapolis, IN) and 0.1% DNase I (Sigma Chemicals, St. Louis, MO) in PBS for 1 h at 37°C, then resuspended into 64% Percoll solution (density, 1.08 g/ml). This mixture was then overlayed with Percoll solutions of decreasing densities (1.06, 1.05, 1.04, 1.03, and 1.01 g/ml) and centrifuged at 600 × g for 30 min at room temperature. In these experiments, the interface from the top layer, the lowest interface, and the pellet were discarded and the remaining layers 2 through 5 were pooled for use as effectors in the DNA fragmentation assay. Cells obtained from these layers consisted of large cells and had previously been characterized as predominantly a trophoblastic phenotype (16). No difference in trophoblast recoveries was observed between the wild-type (wt/gld) or Fas ligand (Fas-l)-deficient (gld/gld) strains of mice.

DNA fragmentation assay

Determination of cell death was made using the DNA fragmentation test that has previously been described (17). Briefly, 1 × 10^6/ml target cell lines (L1210, a DBA/2 lymphocytic leukemia that does not express Fas; L1210 Fas, that has been transfected with the Fas gene; d11S, a mouse T cell hybridoma that does not express Fas; 18) or 2B4.11, a mouse T cell hybridoma that does expresses Fas (19)) were labeled with 5 μCi/ml [3H]thymidine for 3 h at 37°C. Labeled targets were cultured in triplicate at 5 × 10^5 cells/well with titrated numbers of effector cells (either trophoblasts or control cell lines) in 96-well U-bottom plates for 4 h at 37°C. The results are expressed as percentage of cell death and is calculated by: [(C - E)/C] × 100 = % cell death, where C = cpm of cells cultured in the control (i.e., medium alone) and E = cpm of cells cultured in experimental conditions.

T cell proliferation assays

1 × 10^5 lymph node cells were cultured in 96-well flat-bottom plates with titrated numbers of T-depleted irradiated (3000 rad) spleen cells from either male or female C57BL/6 mice with 25% anti-TCR culture supernatant obtained from H57. Cultures were maintained for 4 days at 37°C in a 5% CO_2 atmosphere in complete tissue culture medium, which contained RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 20 mM HEPES, 100 μM nonessential amino acids, 1 mM sodium pyruvate, and 25 μM 2-ME. All cultures were supplemented with 20 μl of medium rIL-2. [3H]Thymidine (1 μCi/well) was added during the last 16 h of culture before harvest. At the end of this time, the cells were harvested onto fiberglass filters using a Tomtec 96-well cell harvester (Tomtec, Orange, CT) and counted on an LKB Betaplate counter (Wallac, Gaithersburg, MD).

Results and Discussion

To address the fate of T cells specific for fetal Ags during pregnancy, lymphoid tissues were analyzed from pregnant RAG^+/- (RAG^+/-) mice that express a transgenic TCR specific for a peptide of the male Ag, H-Y, presented by D^b (oH-Y TCRtg). H-2^d, oH-Y TCRtg RAG^+/- pregnant females that had been mated to C57BL/6 males were evaluated at 7, 14, or 18 days of gestation, 5 or 21 days postpartum. Despite the presence of H-Y-specific T cells in these mice, pregnancies were normal with the expected frequency of male offspring (data not shown). Lymphoid tissues from the mothers were harvested, and cells were counted and analyzed by flow cytometry for expression of the clonotypic TCR (recognized by the Ab T370). Interestingly, clonotypic T cells initially decreased but then increased in the spleen by day 14 of gestation before decreasing during late gestation (Fig. 1A). By day 18 of gestation, the number of clonotypic T cells in pregnant females had decreased by 42% (9.8 ± 2.1 × 10^6 vs 16.8 ± 2.9 × 10^6), and by 5 days postpartum, these females had only 34% of the number of T cells observed in nonpregnant transgenic females (5.8 ± 0.6 × 10^6). Similar to the spleen, the number of clonotypic T cells in the lymph nodes initially began to drop at 7 days of gestation, then expanded by gestational day 14 before decreasing (Fig. 1B). The number of clonotypic T cells was 25% lower (3.0 ± 0.7 × 10^6 vs 4.2 ± 1.1 × 10^6) at day 18 of pregnancy, and by 5 days postpartum, only 50% of the clonotypic T cells observed in nonpregnant controls were detected (2.0 ± 0.5 × 10^6). The apparent decrease in the number of T cells could not be explained by TCR or coreceptor down-regulation in response to antigenic stimulation, as the number of Thy-1-expressing and CD8-expressing T cells decreased equivalently to the number of clonotypic T cells.
nulliparous controls (Fig. 1A and B). To determine when the number of fetal-reactive T cells was restored to normal levels, we evaluated lymphoid tissues from parous females up to 3 wk postpartum. Even after 21 days, the number of clonotypic T cells was still only 50% of the nulliparous controls (Fig. 1A and B). There is a slight increase in the number of Thy$^+$ T cells detectable at 21 days postpartum. Possibly, this increase may reflect freshly exported T cells from the thymus. However, the long term decrease observed in T cells is consistent with the occurrence of clonal deletion.

To address whether the decrease in clonotypic T cells is Ag specific, lymphoid cells from pregnant oH-Y TCRtg RAG−/− mothers that were either H-2$^b$ or H-2$^d$ were compared. H-2$^d$ TCR transgenic mice serve as a control for Ag specificity because they do not express the D$^b$ molecule that would present the H-Y peptide. In marked contrast to H-2$^b$ pregnant females, which showed a decrease in clonotypic T cells, H-Y-specific splenic T cells increased more than twofold in H-2$^d$ pregnant females compared with nonpregnant females (Fig. 1C). This increase appears to be a nonspecific effect of pregnancy, since similar increases in T cells during pregnancy were observed in cytochrome-c-specific TCR transgenic mice (data not shown). When compared 5 days postpartum, the number of H-Y-specific T cells in the parous H-2$^d$ females remained higher than in nulliparous controls, whereas there were even fewer clonotypic T cells in the H-2$^b$ parous females. This decrease could not be accounted for by relocalization of H-Y-specific T cells to the placenta. Analysis of placental tissues from H-2$^b$ pregnant did not reveal increased numbers of T cells compared with placental tissues from H-2$^d$ pregnant (data not shown). In addition, the fate of H-Y-specific T cells in day 14 pregnant RAG+/− females was examined, since these mice also possess nonclonotypic T cells that serve as an internal control to assess the specificity of changes observed in the clonotypic population (Fig. 1D). While significant decreases are observed in the CD8$^+$ clonotypic T cells of both the spleen (9.2 ± 2.4 × 10$^6$ vs 4.6 ± 0.6 × 10$^6$) and lymph nodes (7.9 ± 1.2 × 10$^6$ vs 4.0 ± 0.6 × 10$^6$) compared with nonpregnant controls, no decrease was observed in either the CD4$^+$ or CD8$^+$ nonclonotypic population. Together, these findings demonstrate that fetal-reactive T cell are specifically deleted during pregnancy.

To address the origin of the cells mediating deletion, we examined the fate of maternal T cells from day 14 H-2$^d$, oH-Y TCRtg RAG+/− females that had been mated with either C57BL/6 (H-2$^d$) or BALB/c (H-2$^d$) males. When we assessed the number of clonotypic T cell in H-2$^d$ females bred to H-2$^b$ males, we detected a 60% decrease in the lymph node and a 48% decrease in the spleen (Fig. 2A). However, this decrease was not observed in H-2$^b$ females that were bred to H-2$^d$ males. Since the only source of APCs expressing D$^b$ is the H-2$^{bd}$ fetus, these results demonstrate that deletion of H-Y-specific T cells can result from an encounter with fatally derived cells. However, maternal presentation of shed fetal Ag is also a possibility that cannot be excluded as a mechanism of tolerance induction in the H-2$^d$ females.

Ligation of Fas, a member of the TNF receptor (TNFR) family, rapidly induces apoptosis in activated T cells and has been proposed to play an important role in peripheral T cell tolerance (20–22). The immune-privileged status (i.e., protection against immune attack after allotransplantation) of several tissues has been attributed to the expression of Fas-I (23, 24). Interestingly, Fas-I is expressed on placental trophoblasts that form the physical interface between the mother and the fetus (Refs. 25 and 26; and M.S.V., personal observation). The fact that the placenta is considered an immune-privileged tissue, together with the fact that murine trophoblasts can express MHC-encoded class I (27), raises the intriguing possibility that clonal deletion may result from the encounter of activated fetal-specific T cells with placental trophoblasts. We, therefore, investigated whether trophoblasts may be able induce cell death in two mouse T cell lines, 2B4.11 and d11S, that vary in expression of Fas. 2B4.11 (Fas$^-$) was killed by purified trophoblasts from the placenta of day 18 pregnant mice, whereas d11S (Fas$^+$) (Fig. 2B) was not. However, 2B4.11 was not killed by trophoblasts isolated from placentas that are homozygous for the gld mutation and therefore express nonfunctional Fas-I. To eliminate the possibility that other differences between the cell lines were responsible for the susceptibility to trophoblast-induced killing, purified trophoblasts were tested for the ability to induce cell death in two mouse cell lines that varied only in expression of Fas (18). In Figure 2C, L1210Fas is very efficiently killed by trophoblasts from mice that have normal Fas-I expression, whereas...
isolated from placenta homozygous for the gld mutation. L1210 is not. Furthermore, L1210Fas is not killed by trophoblasts a day 18 pregnant or nonpregnant female responsive to antigenic challenge. 13 FIGURE 3. demonstrating that these cells were quite capable of responding to stimuli. Lymph node and spleen cells from day 18 pregnant and nonpregnant control mice were analyzed for CD8 or T370 expression. In the histograms, the thin line indicates expression in the nonpregnant control, and the thick line indicates expression in the day 18 pregnant mouse.

These experiments reveal that those T cells that have not been deleted have nonetheless been tolerized and are Ag nonresponsive. This does not appear to be a case of “classical” anergy, because the T cells are unresponsive to antigenic stimulation even in the presence of IL-2. T cell responsiveness to TCR stimulation via Ab cross-linking may occur because high avidity interactions can overcome nonresponsiveness. However, nonresponsiveness did not correlate with either TCR or coreceptor down-regulation (Figs. 1, A and B, and 3C). as has been reported in other model systems of peripheral tolerance (3, 8) including pregnancy (28).

It has been demonstrated that clonal deletion can occur in the peripheral immune system (3, 29) when T cells are exposed to high, nonphysiologic concentrations of Ag. This study reports the disappearance from the peripheral immune system of H-Y-reactive T cells when fetal Ag is encountered during pregnancy. Unlike the previously described systems, H-Y would be present in the maternal immune system at lower, more physiologic levels. Studies using a sensitive PCR system for the detection of fetally derived H-Y + cells in various tissues of the maternal immune system demonstrate that circulating H-Y + fetal cells occur infrequently (30). Furthermore, the concentration of H-Y Ag encountered by maternal T cells, via either shed fetal proteins or circulating fetal cells, is variable between mothers depending on the number of male fetuses present in each litter. Nevertheless, the degree to which fetal-reactive T cells are eliminated was consistent, revealing an exquisite sensitivity of the immune system to peripheral Ags.

The mechanisms by which peripheral tolerance is maintained have been shown to include clonal deletion (2–4), clonal unresponsiveness or anergy (5–7), and down-regulation of TCR or coreceptors (3, 8, 9), although the factors that determine which mechanism is invoked are as yet unclear. Recently, Tafuri et al. found that T cells from K b-specific TCR transgenic mice mated to K b-expressing males down-regulated CD8 coreceptors during pregnancy, resulting in reversible functional unresponsiveness to the paternal Ags (28). Our results demonstrate that transient coreceptor down-regulation is not the only mechanism by which maternal T cells are tolerized to fetal Ags and that mechanisms with more long-term effects are active as well. Differences in the avidity of the TCR for the MHC/peptide complex, the expression and presentation of the target Ag, the nature of the APC, or the microenvironment in which the T cell encounters Ag may ultimately dictate the mechanism of tolerance that is invoked. One intriguing possibility to explain the existence of multiple tolerance mechanisms in our system is that presentation of fetal Ags by APCs of either fetal or maternal origin may evoke different mechanisms. Experiments are currently being performed to address this issue. Furthermore, although it has been suggested that chronic exposure to Ag results in down-regulation of TCR and/or CD8 (11–13), we did not observe down-regulation of either TCR or coreceptors on fetal-specific T cells during pregnancy, despite the fact that coreceptor down-regulation has been observed in other models of peripheral tolerance using the aH-Y TCRtg mice (3, 13). This finding would argue that coreceptor down-regulation may not be simply the result of chronic stimulation but, potentially, Ag concentration and route of exposure as well.

In conclusion, this work demonstrates that clonal deletion can occur at physiologic Ag concentrations and can serve as a mechanism of peripheral tolerance. Moreover, maternal T cell recognition of fetal Ags can lead to Ag-specific tolerance. Thus, not only does the maternal immune system respond to the fetus, but more importantly, it is actively tolerized by it. Interestingly, these results reveal that there are multiple mechanisms of peripheral tolerance induction during pregnancy and that those cells that are not clonally deleted become unresponsive to antigenic challenge. The
fact that multiple mechanisms of tolerance are involved in response to this “natural” antigenic challenge may be indicative of the critical importance that mature T cells be tolerant of pregnancy-related and other developmentally regulated Ags to prevent autoimmune attack and allow the survival of the species.

Acknowledgments

We thank Drs. J. D. Ashwell, R. J. Hodes, A. S. Rosenberg, E. Shacter, and E. W. Shores for critical review of the manuscript. We also thank Dr. E. W. Shores for providing the TCR transgenic mice, Dr. J. Zuniga-Pflucker for providing the RAG-2−/− mice, and Ms. Jan Lee for assistance in the breeding and typing of the transgenic mice.

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