Human T-Cell Development in SCID-hu Mice: Staphylococcal Enterotoxins Induce Specific Clonal Deletions, Proliferation, and Anergy

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SCID-hu mice provide an in vivo model for studying the events of normal intrathymic human T-cell development and differentiation. We injected SCID-hu mice with staphylococcal enterotoxins (SE) and determined their effects on the development and responsiveness of human T-cell populations defined by their expression of CD4 and CD8, and the type of Vβ molecule in their T-cell receptors. After single intraperitoneal injections of SEB or SEE, we observed specific effects on thymic T cells expressing a cognate Vβ T-cell receptor (TCR) (Vβ 12.1 in the case of SE-treated SCID-hu mice and Vβ 8.1 in the case of SEE-treated mice) using both immunohistochemical staining of thymic frozen sections and flow cytometric analyses. An injection of SEB resulted in a 32% decrease in the total percentages of Vβ 12.1+ cells in thymic sections after 2 days, with the greatest effect seen in the medulla, without a demonstrable effect on Vβ 5.2/5.3+ or Vβ 8.1+ cells. Fluorescence-activated cell sorter analysis demonstrated that TCRhi thymocytes expressing a cognate Vβ TCR declined transiently by 35% to 45% 1 to 2 days after the injection of SE. Analysis of thymic subpopulations showed decreases in the TCRhi CD4+8+ and CD4+8- cells and an increase in TCRlo CD4-8+ cells. Multiple injections of SE resulted in 50% to 60% decreases in cognate Vβ TCR+ CD4-8+ populations. Thymocytes prepared from SE-treated SCID-hu mice demonstrated specific anergy to the SE to which they had previously been exposed in vivo, but had a normal proliferative response to other superantigens in an in vitro assay. In contrast to the effects on thymic T cells, single injections of SE resulted in a twofold increase in the total numbers of circulating CD4+8+. CD4-8+ human T cells and a fourfold to eightfold increase in T cells expressing a cognate Vβ TCR. Using SE as superantigens in SCID-hu mice, we have been able to induce antigen-specific clonal deletions, anergy, and proliferation of human T cells.

The thymus is a site of clonal deletion of self-reactive T cells and the positive selection of T cells that are capable of recognizing antigens in the context of self-major histocompatibility complex (MHC). The process of clonal deletion to self-antigens has been studied in mice that constitutively express proteins derived from endogenous mammary tumor virus (MTV) genes (minor lymphocyte substance [MLS]; endogenous superantigens) and in weanling mice exposed to repeated doses of staphylococcal enterotoxins (SE; exogenous superantigens). These bacterial and virally encoded proteins are termed "superantigens" because their exposure to mature T cells results in a massive, polyclonal proliferative response of clonotypic T cells that express the same Vβ chain in their T-cell receptor (TCR). In contrast to the effect on mature, peripheral T cells, the exposure of immature T cells in the thymus to endogenous or exogenous superantigens results in a marked reduction in the number of cells that express a cognate TCR-Vβ capable of binding to the respective superantigen compared with control mice that lack expression of the endogenous superantigen or who have not been exposed to SE. Intrathymic transfer of sorted, mouse T-cell populations at different stages of development indicated that positive selection begins at the CD4+8+ TCRβ+ blast stage, resulting in increased frequency of cells committed to the CD4 or CD8 lineage that express intermediate amounts of TCR, whereas negative selection occurs at the same phase of the transition of these cells to virgin CD4 or CD8 thymic T cells expressing high levels of TCR. While the mitogenic effect of exogenous superantigens has been observed in vitro using human T cells, the processes of intrathymic clonal deletion and the induction of tolerance have not been extensively studied. Selective deletion of Vβ 5.2/5.3+ thymocytes and Vβ 8.1+ thymocytes after the addition of SE or SEE to human thymocytes in organ culture with mouse thymus has been recently reported. Thymocytes from SCID-hu mice engrafted with hematopoietic cells and thymic stroma from different fetal donors are tolerant when challenged in vitro to Epstein-Barr virus (EBV)-transformed lymphoblastoid cells from either fetal donor, and thymocytes reactive to the donor of the hematopoietic cells have been deleted. To date, no evidence for clonal deletion in response to endogenous superantigens occurs in humans, because T cells expressing all the different Vβ genes in their TCR appear to be present in roughly equal frequencies in randomly selected individuals. Understanding the mechanism of clonal deletion will likely be important in furthering our understanding of autoimmune diseases that represent a failure to eliminate self-reactive T-cell populations; in graft-versus-host disease seen in allogeneic bone marrow transplantation (BMT); and in augmenting the graft-versus-leukemia phenomenon in autologous BMT. We have used SCID mice engrafted with fragments of human fetal thymus (SCID-hu) as a model system to study the effects of exogenous superantigens on the intrathymic maturation of human T cells. We find that clonal deletion of human T cells expressing a cognate TCR occurs in SCID-hu mice that have been exposed to a single or to multiple doses of SE. We have observed both antigen-specific anergy and a proliferative effect against SE-exposed cells. This suggests that SCID-hu mice provide a useful model for studying the mechanisms of clonal deletion in human T cells.

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response in the thymocytes and mature T cells from SE-treated SCID-hu mice.

**MATERIALS AND METHODS**

**SCID and SCID-hu mice.** C-B 17 scid/scid mice were obtained from Leonard Shultz (Jackson Labs, Bar Harbor, ME) and maintained under specific pathogen-free conditions in the animal facility of the Division of Radiobiology. The mice received a suspension of Trimethoprim sulfamethoxazole in their drinking water three times a week. SCID-hu mice were created using fetal tissues from 16- to 20-week gestations (obtained after therapeutic abortions), as previously described. Use of fetal tissue for research purposes was consented to by the respective patients after their decision to undergo the operative procedure and approved by an institutional review board. Small fragments of fetal thymus and liver (a source of hematopoietic stem cells) were placed beneath the left kidney capsule. Engrafted SCID-hu mice showed the growth of human thymic tissue to more than 100 times the original volume in more than 90% of the cases and the appearance of CD4+8- and CD4-8+ cells in the peripheral blood of the mouse. Approximately 15 SCID mice were engrafted with fragments from each fetal thymus; in each individual experiment, both the treated and control mice were taken from a group of mice that had been engrafted with tissue from the same fetus. The blood of SCID-hu mice was obtained by quickly bleeding the mice (after they had been killed) into tubes containing 5 mmol/L HEPES-buffered Hanks Balanced Salt Solution (HBSS), pH 7.4, containing 0.02% sodium azide and 5% vol/vol heat-inactivated newborn calf serum (HBSS/NCS) and 10 U/mL heparin. The mononuclear cell fraction was obtained after centrifugation over a Ficoll step gradient.

**Administration of SE.** SEB was purchased from Sigma Chemical Co (St Louis, MO). SEE, and SEC3, SED were purchased from Toxin Technology, Inc (Madison, WI). SEE and SED were reconstituted with distilled water to concentrations of 5 mg/mL and stored in aliquots at −20°C. SCID-hu mice received intraperitoneal (IP) injections of SEB or SEE diluted in HBSS/NCS; control mice received injections of HBSS/NCS alone. Some SCID-hu mice received multiple injections of enterotoxins on Monday, Wednesday, and Friday of each week for a total of seven to nine doses before being killed for analysis. SE administration in doses up to 20 µg did not result in any discernable ill effects on the health of the SCID-hu mice.

**Immunohistochemical analysis of Vβ expression on thymic grafts from SCID-hu mice.** Tissue samples were frozen in Tissue-Tek OCT embedding medium (Miles Scientific), stored at −70°C, and cut into thin sections. Immunohistochemistry was performed using a biotin-streptavidin technique previously described. Briefly, cryostat sections were fixed in acetone at 4°C for 10 minutes. Sections were incubated with unlabeled monoclonal antibody (MoAb) to Vβ 5.2 + 5.3, Vβ 8.1, or Vβ 12.1 (T Cell Sciences, Cambridge, MA). Sections were sequentially incubated with appropriate dilutions of biotin conjugated goat antimouse (Jackson Labs). Immunohistochemical analysis of Vβ expression on thymic grafts from SCID-hu mice. Murine antibodies to human CD3, CD4, and CD8 were obtained from Becton Dickinson (Sunnyvale, CA). Antibodies to the human Vβ chains of the TCR were purchased from T Cell Sciences. Cell surface staining and flow cytometric analysis of lymphoid cells was performed as previously described. Briefly, cell suspensions of thymocytes from human thymus xenografts were preincubated with normal mouse serum and then with fluorescently labeled antibodies to the Vβ chain of the TCR, biotinylated anti-CD8, and phycoerythrin-conjugated anti-CD4 at 4°C for 30 minutes in HBSS/NCS in a 50 to 100 µL volume. Control tubes were incubated with a fluorescently labeled mouse IgG in combination with the anti-CD4 and anti-CD8 antibodies described above. Multicolor immunofluorescent analyses of cell suspensions in HBSS/FACS + 1 µg/mL propidium iodide (PI) were performed by a highly modified dual-laser fluorescent-activated cell sorter (BD FACS System; Becton Dickinson) equipped with a four-decade logarithmic amplifier. Dead cells were excluded from the analysis by normal-scatter gating method and by setting an electronic gate to exclude PI-positive cells. Computer-generated contour plots from fluorescent measurements of 50,000 viable cells/sample were used to present the two-color staining patterns. The number of contour lines drawn in a particular area represents the frequency of cells exhibiting a given level of fluorescence, in which the area contained between any two lines represents 5% of the total cell population. The contour plots are labeled according to the established names of the molecules detected by the particular antibodies (eg, CD4 and CD8). Density plots of Vβ expression on the CD4+8- and CD4-8+ subsets were obtained by electronically selecting the relevant subpopulation and plotting the density profiles (relative number of cells) of Vβ+ cells out of the total CD4+ or CD4- subsets. The percentage of Vβ+ cells in each subpopulation was determined by integrating the area under the curve for those cells expressing more than the background level of fluorescent staining obtained using the fluorescein isothiocyanate (FITC)-labeled control mouse IgG. The mean values for the percentage of Vβ+ cells in each subpopulation at different time points after SE administration were calculated along with the standard deviation (SD) and the standard error of the mean (SEM = SD/square root [number of elements in the data set]) for each group of data. The significance of the difference between the relevant mean values for the percentages of Vβ+ cells in different subpopulations from the group of control mice and mice that had received an injection of SE were calculated according to the two-tailed Student's t-test. Enterotoxin-induced proliferation of thymocytes from SCID-hu mice. Single-cell suspensions from thymus grafts were obtained sterciley from treated or control mice were washed two times in plain Iscove's modified Dulbecco's medium (IMDM; Sigma). The cells were resuspended to 5 × 10^6/mL in IMDM supplemented with 2% vol/vol normal mouse serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 µg/mL indomethacin, 5 × 10^-5 mol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, 5 µg/mL transferrin, 5 ng/mL sodium selenite, 5 µg/mL insulin, and 2 mmol/L glutamine. Cells (5 × 10^6) in 100 µL were added to wells in round-bottom tissue culture plates (Costar, Cambridge, MA), along with a selected toxin (Toxin Technology) at a final concentration of 1 µg/mL. Cells were incubated for 2 days at 37°C and then pulsed with 1 µCi ^3H-thymidine (Amersham, Arlington Heights, IL) for an additional 18 hours. Cells were harvested by aspirating the cell suspension onto glass fiber filters and washing the filters with distilled water. Each experimental point represents the mean of 8 to 12 wells.
RESULTS

Effects of SEB administration on Vβ TCR, CD4, and CD8 expression on SCID-hu thymocytes. We first studied the expression of Vβ 5.3/5.3, 8.1, and 12.1 in thymic frozen sections from SEB-treated SCID-hu mice using an immunohistochemical technique. Previous work had established that SEB is mitogenic for peripheral human T cells expressing Vβ 12.1, and that administration of SEB to neonatal mice results in the intrathymic deletion of cells expressing the corresponding cognate murine TCR. We found that 41 per 1,000 thymocytes were positive after staining with the Vβ 12.1 antibody in untreated thymus grafts. Two days after SEB administration, the number of Vβ 12.1+ cells had declined to 28 per 1,000 thymocytes, a 32% reduction; 4 days after SEB injection, increased it to 57 per 1,000 thymocytes, a 24% increase (Fig 1). A larger reduction in

![Image of Vβ expression in thymus xenografts](https://example.com/image)

Fig 1. Immunohistochemical analysis of Vβ 12.1 expression in thymus xenografts from SEB-treated SCID-hu mice. Frozen sections of thymus grafts from an untreated control SCID-hu mouse and mice 2 and 4 days after SEB injection were stained with antibodies to Vβ 5.2/5.3, Vβ 8.1, or Vβ 12.1 as described in Materials and Methods. The location of the cortex and medulla are indicated by the small letters to the right.
Vβ 12.1 expression 2 days after SEB injection was seen in the medulla (a 44% decrease) compared with the cortex (a 22% decrease). No significant effect on the percentages of Vβ 5.2/5.3+ or Vβ 8.1+ cells was seen (not shown). Thymic xenografts from untreated SCID-hu mice contained human thymocytes whose numbers varied over a 2 log range, with a mean of \(83 \times 10^6\) (±SD of \(87 \times 10^6\)); administration of 2 µg SEB did not result in any significant decrement in the mean number of cells compared with control mice injected with buffer alone (Fig 2A). Injections of more than 2 µg of SEB resulted in a more than 50% decrease in the average numbers of viable human thymocytes that could be recovered from treated SCID-hu mice compared with SCID-hu mice engrafted with the same fetal thymic tissue, but not exposed to SE (data not shown). The remainder of experiments described below used a standard dose of 2 µg SEB.

To quantify the effects of SE on levels of TCR expression in SCID-hu thymocytes, we used multiparameter flow cytometric analysis to assess the expression of CD4, CD8, Vβ 5.2/5.3, Vβ 8.1, and Vβ 12.1. In a compilation of data from experiments involving eight different fetal donors and 181 SEB-treated SCID-hu mice, the mean percentage (±SD) of CD4+8− cells in untreated mice was 12% ± 7.5% and remained stable over 6 days (mean value at 6 days of 13% ± 1.7%), whereas the fraction of CD4+8+ cells in control mice was 5.2% ± 5.6% and remained stable over 6 days (mean value at 6 days of 4.7% ± 0.3%). A representative experiment is shown in Fig 2B, with the gates used to define the CD4+8+ and CD4+8− populations indicated by the rectangles, and the fraction of cells contained within these populations indicated by the numbers within the rectangles.

Flow cytometric analysis of Vβ 5, 8, and 12 expression on thymocytes from control SCID-hu mice and SEB-treated SCID-hu mice demonstrated a 44% reduction in the percentage of TCRhi cells expressing Vβ 12.1 1 day after SEB injection, followed by a 100% increase in the percentage of Vβ 12.1hi cells at 5 days without a demonstrable effect on levels of Vβ 5.2/5.3hi or Vβ 8.1hi expression (Fig 3). The electronic gate used to delimit the Vβ+ cells from the negative population was defined by the staining profile of thymocytes using an FITC-labeled control mouse IgG.
Fig 3. The effect of SEB administration on the relative levels of Vβ 5.2/5.3, Vβ 8.1, and Vβ 12.1 expression on SCID-hu thymocytes. Flow cytometry was performed on thymocytes from untreated and SEB-treated SCID-hu mice using FITC-conjugated antibodies to either Vβ 5.2/5.3, Vβ 8.1, or Vβ 12.1. The abscissa represents the level of fluorescence staining (log scale), while the ordinate represents the relative number of cells (arbitrary units) with a given level of fluorescence. A representative profile obtained using a control FITC-conjugated mouse IgG1 (---) is shown in the last panel in the middle row. The density profile of fluorescent staining and the percentages of TCR0 and TCRhi cells in the thymus using each of the FITC-conjugated antibodies in control SCID-hu mice and at 1, 3, and 5 days after SEB injection is shown.

When cells from a fragment of the same thymic tissue shown in Fig 1 were analyzed for Vβ expression by flow cytometry, the percentage of Vβ 12.1+ cells was 3.2% in the graft from the untreated SCID-hu mouse, declined to 1.8% in a graft from a SCID-hu mouse 2 days after SEB administration, and then increased to 3.8% 4 days after SEB administration. When an electronic gate was set to count the cells expressing the highest level of Vβ 12.1 (eg, the TCRhi cells as shown in Fig 3), there was an even greater effect of SEB administration on Vβ 12.1 expression: a 68% decline from 2.5% positive cells in control mice to 0.8% positive cells in grafts from SCID-hu mice 2 days after SEB administration. Because the most pronounced effect of SEB administration was seen on cells expressing the highest levels of TCR and in medullary cells, both characteristics of thymocytes at the most mature stage of intrathymic development, we examined the levels of TCR expression on thymocytes in the (phenotypically mature) CD4+8- and CD4-8+ subpopulations. Because the total number of human thymocytes varied significantly among different thymic xenografts (Fig 2A), it was difficult to compare the effect of SE administration on the absolute numbers of Vβ+ T cells among SCID-hu mice that received different treatments. We therefore calculated the percentage of cells in the CD4+8- and CD4+8+ thymic subpopulations expressing Vβ 5.2/5.3, Vβ 8.1, or Vβ 12.1 from control mice and in mice that had received a single injection of SEB. The staining intensity for Vβ expression in these subpopulations was generally similar to the Vβhi cells shown in Fig 3. Incubating the thymocytes with a control, FITC-labeled mouse IgG1, in combination with CD4 and CD8 antibodies, resulted in less than 0.02% of CD4+8- or CD4+8+ cells, with a fluorescein signal greater than the lower gate for fluorescein-positive cells indicated in Fig 3 (not shown). The percentages of CD4+8- and CD4+8+ cells expressing
either Vβ 5.2/5.3, 8.1, or 12.1 from eight experiments involving 181 SCID-hu mice are shown in Fig 4. We observed 52% and 29% decreases in the mean percentage of CD4+8- thymocytes expressing Vβ 12.1 at 24 and 48 hours, respectively, compared with the corresponding population of Vβ 12.1 CD4+8- thymocytes from untreated SCID-hu mice (P < .001 and P < .05) and increases of 39%, 37%, and 45% in the mean percentages of Vβ 12.1+ CD4+8- thymocytes at 48, 72, and 96 hours, respectively, after SEB administration (P < .05, P < .05, and P < .05). The expression of Vβ 5.2/5.3 and 8.1 in CD4+8- and CD4-8+ thymic subpopulations was not significantly altered by exposure to SEB, with the exception of increases in the percentages of CD4+8- and CD4+8+ thymocytes expressing Vβ 5.2/5.3 and Vβ 8.1 of 38% to 94% to 6 to 7 days after SEB administration (P < .2 to P < .001). Multiple injections of SEB resulted in a 60% decrease in the fraction of Vβ 12.1+ CD4+8- thymocytes compared with control SCID-hu mice (P < .05) and a 120% increase in Vβ 5.2/5.3 CD4-8+ thymocytes (P < .05) (Fig 5). The differences observed after multiple injections of SEB occurred without significant effects on the total numbers of thymocytes recovered from SCID-hu mice. Lower doses of SEB pro-

Fig 4. The time course of the effect of a single SEB injection on the expression of Vβ 5.2/5.3, 8.1, and 12.1 in CD4+8- and CD4-8+ cells obtained from SCID-hu mice. Thymocytes from SEB-treated SCID-hu mice were analyzed according to the legend for Fig 3. The percentages of CD4+8- and CD4-8+ cells expressing Vβ 5.2/5.3, Vβ 8.1, and Vβ 12.1 from multiple experiments are shown. The solid line connects the mean ± SEM values calculated from analyses performed on 3 to 20 individual SCID-hu mice at each time point. Significant differences between the mean percentages of positive cells from control SCID-hu mice (day 0) and the mean values obtained at various times after SEB administration are represented by the following symbols shown above the respective time points: (○) P < .05; (△) P < .02; (+) P < .01; (*) P < .001.

Fig 5. The effect of multiple injections of SEB on the expression of Vβ 5.2/5.3, 8.1, and 12.1 on CD4+8- and CD4-8+ thymocytes obtained from SCID-hu mice. SCID-hu mice received eight injections of 2 μg SEB over 18 days. Thymocytes from SEB-treated SCID-hu mice were analyzed according to the legend for Fig 3. The mean values ± SD for the percentages of Vβ 8.1+ and Vβ 12.1+ cells in the CD4+8- and CD4-8+ populations were determined. Each data point represents the mean of analyses performed on six individual SCID-hu mice. Significant differences between the mean percentages of positive cells from control SCID-hu mice (left) and the mean values obtained after multiple injections of SEB (right) were determined and are represented according to the legend for Fig 4.

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duced qualitatively similar effects on Vβ expression, but of a smaller magnitude, than the standard doses of 2 μg that we have used throughout the rest of this work.

Effects of SEE administration on Vβ TCR, CD4, and CD8 expression on SCID-hu thymocytes. We next studied the effects on human T-cell development in SCID-hu mice of administering SEE, a staphylococcal enterotoxin that is known to have mitogenic effects on mature human T cells expressing TCR Vβ 8.1. The effect of a single 0.5 μg SEE injection was to increase the fraction of cells in the CD4+8- and CD4-8+ populations to 20% ± 3.7% and 7% to 1.2%, respectively, in mice that had been treated 7 days earlier with SEE, compared with 13% ± 4% and 4% ± 1.4%, respectively, in untreated control SCID-hu mice (P < .001 and P < .001, respectively), and caused a gradual, slight decrease in the average numbers of thymocytes that were recovered from the thymic xenografts: 98.5 × 10^6 ± 26 × 10^6 in control mice, 131 × 10^6 1 day after SEE administration, 44 × 10^6 2 days after, 92 × 10^6 3 days after, 37 × 10^6 5 days after, and 34 × 10^6 7 days after. Larger doses of SEE resulted in a greater cytopathic effect. The effect of SEE administration on Vβ 8.1 expression in the CD4-8+ and CD4+8- subpopulations from a representative experiment is shown in Fig 6; note the decrease in the Vβ 8.1+ cells in both subpopulations and an increase in cells that express intermediate levels of Vβ 8.1 in the CD4+8- subpopulation. The mean values from four experiments involving 80 SCID-hu mice are shown in Fig 7. We observed a transient 33% decrease in the mean percentage of CD4+8- cells expressing Vβ 8.1 at 24 hours (P < .05) and a corresponding 94% increase in the mean percentage of CD4-8+ cells expressing Vβ 8.1 at 48 hours (P < .02), with minimal effects on the Vβ 5.2 and Vβ 12.1 populations. Multiple injections of SEE resulted in a 49% decrease in the mean percentage of CD4+8- cells expressing Vβ 8.1 (P < .01) and a nearly complete elimination of CD4+8- or CD4-8+ cells expressing Vβ 5.2 (P < .001), without any significant change in the percentages of CD4+8+ or CD4+8- cells expressing Vβ 12.1 (Fig 8).

Development of antigen-specific anergy in thymocytes from SCID-hu mice. The effect of a single injection of SE was transient and partial with respect to the elimination of T cells expressing the cognate TCR; 4 days after SEE injection the percentages of CD4+8- and CD4-8+ T cells expressing Vβ 12.1 had returned to control values. We tested whether thymocytes that expressed the cognate TCR after SE administration were capable of a proliferative response when rechallenged to the corresponding SE in vitro. Thymocytes obtained 4 days after SE administration to SCID-hu mice failed to proliferate in vitro in the presence of SEB, but had a normal proliferative response to SEE and SEC3 and an intermediate proliferative response to SED (Fig 9). Similarly, thymocytes obtained from SEE-treated mice had a diminished proliferative response in vitro to SEE; 37,015 cpm were incorporated by thymocytes obtained 1 day after SE administration and cultured in vitro with SEE, compared with 174,326 cpm incorporated in control thymocytes cultured in vitro with SEE and 61,762 cpm incorporated by thymocytes obtained from SCID-hu mice 1 day after SE administration and cultured with SEC3.

Analysis of human T cells in the blood of SCID-hu mice. The effect of SEB administration was to cause a twofold increase in the small percentages of phenotypically mature (CD4- and CD8+) human T cells when the blood of SCID-hu mice was analyzed by flow cytometry (Table 1). Within the CD4+ and CD8+ subpopulations, rare cells expressing specific Vβ receptors could be detected in control mice and those that had been exposed to SEB. Three to 4 days after SEB injection, we observed fourfold increases in the fractions of Vβ 12.1+ CD4-8+ and sixfold increases in Vβ 12.1+ CD4-8+ cells (P < .05) compared with the mean percentages of CD4+ and CD8+ cells expressing Vβ 12.1 from control SCID-hu peripheral blood lymphocytes (PBL) (Table 1). Significantly increased percentages of T cells expressing Vβ 5.2/5.3 and 8.1 were not seen after SEB administration. Multiple injections of SEB did not result in larger percentages of detectable Vβ 12.1 cells in the circulation of the mouse. When the blood of SCID-hu mice that had received injections of 0.5 μg of SEE 5 days earlier was analyzed for Vβ TCR, CD4 and CD8 expression, we observed that the mean percentages ± SD of CD4+8- and CD4-8+ cells increased to 0.35% ± 0.06% and 0.68% ± 0.006%, respectively, in SEE-treated SCID-hu mice. The effect of SEB administration on the percentages of CD4+8+ and CD4-8+ T cells expressing Vβ 12.1 was analyzed by flow cytometry (Table 1). Within the CD4+ and CD8+ subpopulations, rare cells expressing specific Vβ receptors could be detected in control mice and those that had been exposed to SEB. Three to 4 days after SEB injection, we observed fourfold increases in the fractions of Vβ 12.1+ CD4-8+ and sixfold increases in Vβ 12.1+ CD4-8+ cells (P < .05) compared with the mean percentages of CD4+ and CD8+ cells expressing Vβ 12.1 from control SCID-hu peripheral blood lymphocytes (PBL) (Table 1). Significantly increased percentages of T cells expressing Vβ 5.2/5.3 and 8.1 were not seen after SEB administration. Multiple injections of SEB did not result in larger percentages of detectable Vβ 12.1 cells in the circulation of the mouse. When the blood of SCID-hu mice that had received injections of 0.5 μg of SEE 5 days earlier was analyzed for Vβ TCR, CD4 and CD8 expression, we observed that the mean percentages ± SD of CD4+8- and CD4-8+ cells increased to 0.35% ± 0.06% and 0.68% ± 0.006%, respectively, in SEE-treated SCID-hu mice.
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Fig 7. The time course of the effect of a single SEE injection on the expression of Vβ 5.3, 8.1, and 12.1 in CD4+8− and CD4−8+ cells obtained from SCID-hu mice. Thymocytes from SEE-treated SCID-hu mice were analyzed according to the legend for Fig 6. The percentages of CD4+8− and CD4−8+ cells expressing Vβ 5.2/5.3, Vβ 8.1, and Vβ 12.1 from multiple experiments are shown. The solid line connects the mean ± SEM values calculated from analyses performed on 3 to 10 individual SCID-hu mice at each time point. Significant differences between the mean percentages of positive cells from control SCID-hu mice (day 0) and the mean values obtained at various times after SEE administration are represented according to the legend to Fig 4.

Fig 8. The effect of multiple injections of SEE on Vβ 5.2+/5.3, 8.1, and 12.1 expression on CD4+8− or CD4−8+ thymocytes. SCID-hu mice received eight injections of 0.5 μg SEE over 18 days. Thymocytes from SEE-treated SCID-hu mice were analyzed according to the legend for Fig 6. The mean values and SD for the percentages of Vβ 5.2+, Vβ 8.1+, and Vβ 12.1+ cells in the CD4+8− and CD4−8+ populations were determined. Each data point represents the mean ± SD of analyses performed on six individual SCID-hu mice. Significant differences between the mean percentages of positive cells from control SCID-hu mice (left) and the mean values obtained after multiple injections of SEE (right) were determined and are represented according to the legend for Fig 4.

DISCUSSION

The effects of parenteral administration of SE to SCID-hu mice on human T cells varied according to their stage of development. Four different effects were seen: (1) the intrathymic deletion of CD4+8− cells expressing the cognate TCR after SE administration; (2) an increase in the percentages of CD4+8− cells expressing intermediate levels of the cognate TCR accompanied by a marked reduction of CD4+8− cells expressing high levels of the cognate TCR after a single dose of SE; (3) the development of antigen-
specific anergy on thymocytes obtained from SE-treated mice; and (4) an increase in the numbers of T cells in the peripheral blood of SE-treated SCID-hu mice. Before discussing the specific effects of SE in this system, two technical points deserve comment. First, the fetal tissue used to engraft these mice was heterogeneous, varying in terms of the gestational age of the tissue (16 to 23 weeks), and there were cytogenetic abnormalities (approximately 50% of fetuses had prospectively identified trisomy, of which the majority involved chromosomes 21 or 18). The size of the thymic xenografts varied inversely with the gestational ages of the fetal tissue used for grafting and varied directly with the time between the transplantation of the fetal tissue and its use in the experiments described herein. The total number of thymocytes that could be obtained from a single thymic xenograft in an untreated SCID-hu mouse varied over 2 logs, between 2.4 × 10^6 and 360 × 10^6, with a mean value of 83 × 10^6 ± 86 × 10^6 in experiments involving SEB. When analyzed by flow cytometry, the ratio between CD4^+8^- and CD4^-8+ cells was close to, or slightly above, 2, a value consistent with the relative percentages of CD4^+ and CD8^+ cells in the blood of young children. There was considerable variability in the percentages of CD4^+8^- and CD4^-8+ cells with ranges of 3.3% to 41.6% and 1.6% to 32%, respectively; and mean values ± SD of 12.2% ± 7.5% and 5.2% ± 5.6%, respectively. Despite these variables, the percentages of CD4^+8^- and CD4^-8+ cells expressing a given Vβ chain from control mice were remarkably constant, as were the qualitative effects of SE on cells expressing the cognate TCR receptor in SCID-hu mice engrafted with thymic tissue from different fetal donors. Some of the variation we observed in the quantitative effects of SE on T cells in the SCID-hu system may be due to differences in the HLA haplotype of the fetal tissue xenograft and a consequent variation in the affinity of SE binding to class II molecules; the size of the graft; the gestational age of the fetus; the age of the graft; or the relative percentages of single-positive versus double-positive T cells.

The second technical point is that an effect of SE in this system reflects an effect on the cellular physiology of developing thymocytes rather than modulation or blocking of surface TCR. Control experiments demonstrated that preincubation of human thymocytes with SEB for 30 minutes at concentrations from 0.005 to 0.5 μg/mL had no effect on the subsequent binding of anti-Vβ 12.1 TCR antibody (not shown), indicating that masking or modulation of surface TCR associated with the binding of SE could not account for the specific deletion of Vβ 12.1^+ CD4^+8^- cells we observed. Additionally, the greatest effect of SEB in terms of antigen-specific anergy and deletion of clonal T-cell populations was seen 24 to 96 hours after intraperitoneal injection, whereas thymocytes obtained 6 hours after SEB administration expressed Vβ 12.1 at the

### Table 1. Flow Cytometric Analysis of Peripheral Blood Mononuclear Cells From SCID-hu Mice

<table>
<thead>
<tr>
<th></th>
<th>CD4^+8^-</th>
<th>Vβ^+ CD4^+8^-</th>
<th>CD4^-8+</th>
<th>Vβ^- CD4^-8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.23 ± 0.15</td>
<td>0.36 ± 0.08</td>
<td>0.010</td>
<td>0.009</td>
</tr>
<tr>
<td>Vβ 5.2/5.3</td>
<td>0.003</td>
<td>0.004</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td>Vβ 8.1</td>
<td>0.004</td>
<td>0.006</td>
<td>0.010</td>
<td>0.006</td>
</tr>
<tr>
<td>Vβ 12.1</td>
<td>0.004</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.19 ± 0.11</td>
<td>0.33 ± 0.09</td>
<td>0.015</td>
<td>0.006</td>
</tr>
<tr>
<td>Vβ 5.2/5.3</td>
<td>0.007</td>
<td>0.017</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td>Vβ 8.1</td>
<td>0.017</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td>Vβ 12.1</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td>Day 3/4</td>
<td>0.29 ± 0.10</td>
<td>0.65 ± 0.34*</td>
<td>0.005</td>
<td>0.009</td>
</tr>
<tr>
<td>Vβ 5.2/5.3</td>
<td>0.002</td>
<td>0.016</td>
<td>0.005</td>
<td>0.009</td>
</tr>
<tr>
<td>Vβ 8.1</td>
<td>0.016</td>
<td>0.025*</td>
<td>0.009</td>
<td>0.009</td>
</tr>
<tr>
<td>Vβ 12.1</td>
<td>0.025*</td>
<td>0.038</td>
<td>0.009</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Untreated SCID-hu mice and SCID-hu mice that had been injected 1, 3, or 4 days earlier with a single injection of 2 μg SEB were killed and exsanguinated. Their heparinized blood was diluted with HBSS/NCS and centrifuged over a Ficoll step-gradient. Suspensions of mononuclear cells were analyzed for the expression of CD4, CD8, Vβ 5.2/5.3, Vβ 8.1, and Vβ 12.1 as described in the legend to Fig 4. The percentage of human CD4^-8^- and CD4^-8+ cells was determined, and the mean ± SD values from six to nine SCID-hu mice for each time point is shown. Data from SCID-hu mice injected 3 and 4 days earlier with SEB were combined. The percentage of cells expressing Vβ 5.2/5.3, Vβ 8.1, or Vβ 12.1 in the CD4^-8^- and CD4^-8+ subpopulations was determined.

*The mean percentage of CD4^-8^- cells had increased relative to control mice with a significance of P < .02.
†The mean number of Vβ 12.1^+ CD4^-8+ cells had increased relative to control mice with a significance of P < .05.
EFFECTS OF SUPERANTIGENS ON HUMAN THYMOCYTES

Fig 10. The forward scatter profiles of CD4-8- and CD4+8- cells from the blood of SCID-hu mice. Cells obtained from the peripheral blood of untreated SCID-hu mice (---) or SCID-hu mice that had been treated 3 days earlier with a single injection of 2 μg SEB (--), were analyzed for CD4 and CD8 expression as described in the legends to Fig 2. (A) The forward scatter profiles of CD4+CD8- cells. (B) The forward scatter profiles of CD4+CD8+ cells.

The qualitative effect of multiple injections of SE to human T-cell development was similar to what has been reported in murine systems (in which neonatal mice received multiple SEB injections of up to 100 μg, and deletion of 58% of the total population of mouse thymocytes expressing the cognate Vβ TCR occurred),12 and in mouse thymic organ cultures, in which the addition of SEB to the culture media resulted in the deletion of 45% to 93% of cells expressing the cognate TCR after 4 days.31 However, the magnitude of the reduction of human T cells of mature phenotypes expressing the cognate Vβ TCR was less than that seen in mice, in which case multiple injections of 100 μg SEB resulted in the deletion of more that 90% of the mature (cortisol-resistant) T cells expressing the cognate TCR.12 These differences may reflect a lower efficiency of antigen presentation in the SCID-hu thymic xenografts, heterogeneity of the T-cell population expressing the cognate TCR such that some of the human thymic T cells fail to interact with the relevant SE or the lower doses of SE that we have used in this study compared to earlier studies that described the effect of SE on murine T-cell development, or other, undefined factors in this system. Some of the sequelae of administering SE to SCID-hu mice may also reflect an indirect action of cytokines released by T cells that have bound SE. The addition of SE to short-term human thymocyte cultures at concentrations of less than 1% of what was injected into SCID-hu mice described in this study results in the deletion of more than 90% of T cells expressing the cognate TCR (Waller et al, in preparation), indicating that intrinsic resistance of human T cells to the effects of SE is unlikely. In view of the significant deletions observed after the administration of much smaller doses of SE, human thymic T cells appear to be at least as sensitive to the effects of exogenous superantigens as murine thymocytes.

In view of the lack of any known endogenous superantigen in humans,19,32 the relevance of the present work to understanding normal T-cell development and pathophysiology is in elucidating the sequence of events that likely occur during clonal deletion of CD4+8- T cells in response to self-antigens presented intrathymically. The failure of
such deletion (or anergy) to occur would result in the development of populations of mature, autoreactive T cells, and may be relevant to autoimmune disorders, graft-versus-host disease, and a graft-versus-tumor effect in BMT recipients.

The second effect on T cells that we have observed using this system is a deletion of CD4-8- cells expressing high levels of the cognate TCR accompanied by a relative expansion of CD4-8+ cells expressing intermediate levels of the cognate TCR after a single administration of SE. In SCID-hu mice that received SEB, we noted a significant and sustained increase in the mean percentages of CD4-8+ cells that expressed Vβ 12.1 2 to 4 days after SEB administration and a sharp increase in the mean percentages of CD4-8+ and CD4-8+ cells that expressed Vβ 5.2/5.3 and Vβ 8.1 6 to 7 days after SEB administration (Fig 4). Analysis of the density profiles of the Vβ 5.2/5.3+ and Vβ 8.1+ cells at 6 to 7 days did not indicate a significant expansion of CD4-8+ or CD4-8+ cells expressing low levels of TCR, as was the case in the expansion of the CD4-8+ Vβ 12.1+ population at 2 to 4 days (data not shown). The effect of an injection of SEB on Vβ 5.2/5.3+ and Vβ 8.1+ cells may represent the activity of impurities in the SEB preparation, or an unanticipated effect of SEB on other T-cell populations. In mice, SEB administration leads to a transient increase in CD4-8- and CD4-8- cells expressing the cognate TCR in peripheral lymphoid organs.15 In the human thymic xenografts we have studied, blast cells expressing the cognate TCR were only observed in the TCRh CD4-8+ populations, whereas there was a concomitant transient accumulation of TCRh CD4+8+ cells and deletion of TCRb CD4-8+ cells after the injection of a single dose of SE (Waller et al, in preparation).

A third effect of SE administration was that the thymocytes obtained more than 6 hours after SE administration failed to proliferate in vitro when cultured in the presence of the same SE to which they had been exposed in vivo (Fig 9). The proliferative response of thymocytes from SE-treated SCID-hu mice was normal when they were cultured with other SE, indicating that the anergy observed in vitro was antigen-specific. The percentage of phenotypically mature thymocytes expressing Vβ 12.1+ was equal to (or greater than) that in control mice levels 72 to 96 hours after SEB administration (Fig 4), whereas thymocytes exposed in vivo to SEB remain anergic to subsequent exposure to SEB in vitro. Both clonal deletion and clonal anergy as mechanisms of antigen-specific tolerance have been described in Mls-1b(MTV-) mice that received indirect or direct intrathymic transfer of Mls-1a(MTV+) cells,33,34 and clonal anergy has been seen in mice immunized with either the endogenous superantigen Mls/MTV or SEB. Mls-1b mice immunized with Mls-1a+ cells develop a Mls-1a-specific anergy when subsequently challenged with Mls-1a in an in vitro proliferation assay,35 T cells from mice immunized with SEB develop a specific anergy to the mitogenic effects of SEB in an in vitro proliferation assay.15,36,37 Clonal deletion and clonal anergy may be mediated through different mechanisms with deletion (in the case of Mls-1a), the consequence of intrathymic antigen presentation of MTV gene products by CD5+ B cells, and clonal anergy resulting from antigen presentation by dendritic cells.34 Our data show that clonal deletion occurs in the human thymus in the presence of SE, and support the conclusion that clonal anergy can be observed in human thymocytes obtained from SCID-hu mice exposed in vivo to SE. The brief period during which we have observed clonal deletion in vivo (1 to 2 days) compared with the longer duration of anergy (1 to 7 days) indicates that the failure of cells to proliferate in vitro after in vivo exposure to SE was not a consequence of the absence of cells expressing the relevant cognate Vβ TCR at the latter time points (compare Figs 4, 7, and 9). The different kinetics of deletion and anergy in this system may represent either (1) the intrathymic presentation of SE by the same antigen-presenting cells (APC) over the 7-day period, such that APC induced clonal deletion when high concentrations of antigen were present (days 1 and 2) and clonal anergy when lower amounts of bound antigen were present (days 3 to 7), or (2) antigen presentation by different types of cells: APC with a low binding affinity for SE, or a rapid catabolism of bound SE, that induce clonal deletion when intrathymic concentrations of SE are high; and a second class of APC with high binding affinity for SE (dendritic cells38) or slow catabolism of bound SE that induce clonal anergy when intrathymic antigen concentrations are low. An alternative interpretation of the data from SCID-hu mice is suggested by the fact that the SE-induced deletion in vivo was never complete, and did not exceed 50% at 1 to 2 days after a single injection of SE. It is possible that within the cognate Vβ TCR+ population there exist two discrete subsets of cells: those that interact with SE in vivo and are deleted, and those that fail to interact with SE. Although the total numbers of cells expressing the cognate Vβ TCR were at normal or increased levels 3 to 7 days after SEB administration (Figs 3, 4, and 7), the unresponsive subset of cognate Vβ TCR+ cells could have emerged as a greater fraction of the total cognate Vβ TCR+ population, such that the overall proliferative response of these cells in vitro to SE was substantially less than cells from thymus grafts that had not been exposed to SE. The high efficiency of SE-induced deletion of human thymocytes expressing the cognate Vβ TCR in vitro (Waller et al, in preparation) makes the existence of two populations of thymocytes expressing the cognate Vβ TCR with different responsiveness to SE in vivo unlikely. Thus, SE-treated SCID-hu mice should be an interesting model system in which to study the mechanism by which developing human T cells are tolerized to self-antigens.2,39

The fourth effect of SE administration was that T cells with a mature phenotype, present in the peripheral blood of the SCID-hu mice, increased substantially in response to SE administration. The effect was transient, occurring 3 to 4 days after SE injection (Table 1). A mitogenic effect has been described after in vitro stimulation of human T cells by SE,10,13,16,40 and for Vβ 3+, 8.1+, 8.2+, and 8.3+ murine cells by SEB.12 The increase in circulating human T cells could be due to (1) the egress of increased numbers of T cells from the thymus, (2) a transient increased intravascular survival and accumulation of T cells, (3) an intravascular proliferation of human T cells, or (4) some combination of these factors. While the numbers of detectable human T
cells expressing any specific Vβ TCR in the blood of SCID-hu mice were very small, we observed a reproducible, threefold to sixfold increase in peripheral T cells bearing the cognate TCR (Table 1) 3 to 4 days after SE administration compared with control mice. This was most significant in the CD4-8- subpopulation and occurred at a time when the corresponding populations of thymic T cells expressing the cognate TCR were either decreased (the CD4-8- subpopulation) or increased (the CD4-8+ subpopulation) relative to the same populations in control mice that had not been exposed to SE (Figs 4 and 7). It seems unlikely that the larger number of circulating TCR+ cells observed after SE administration was due to a threefold to sixfold increased thymic production of these cells. We have no data on the intravascular survival of human T cells in SCID-hu mice, but do note that human cells do not accumulate in the blood of SCID-hu mice to any significant degree, despite what we presume to be constant thymic production of T cells. The absolute numbers of human T cells in the blood of SCID-hu mice observed in this study were small, as the CD4-8- and CD4-8+ subpopulations combined comprised less than 1.5% of the low-density mononuclear cells in the blood of SE-treated animals (Table 1) and represented less than 70,000 circulating human T cells (1.5% × 1.5 mL blood/mouse × 3 × 10^6 mononuclear cells/mL), whereas the number of peripheral CD4+ or CD8+ human T cells expressing any particular Vβ TCR was less than 4,000 cells/mouse. One explanation for the observed increase in the numbers of TCR+ cells in the peripheral circulation of SE-treated SCID-hu mice is that SE was acting as a mitogen for the small numbers of human T cells expressing the cognate TCR normally present in the peripheral blood of SCID-hu mice. This hypothesis was supported by a flow cytometric analysis of the size of the human T cells in the peripheral blood of SCID-hu mice, which suggested that T cells from SE-treated mice, being larger than those present in control mice, were activated and/or proliferating (Fig 10).

The relatively rare human T cells in the blood of SCID-hu mice (engrafted with human fetal thymic and liver tissue) proliferate in vitro in response to mitogens, although most of these T cells are CD45Ro+ and their activation by phytohemagglutinin (PHA) is less efficient than the activation of normal human PBL.4 In contrast to the technique of engrafting fragments of fetal tissue into SCID mice we have used in this study,20,21 transplantation of EBV- human PBL intraperitoneally into SCID mice42 or human umbilical cord blood43 leads to the gradual accumulation of CD45Ro+ human T cells that are mitotically active in vivo but anergic in vitro when challenged with SCID spleen cells, PHA, or anti-CD3 (in the absence of exogenous interleukin-2).45 The differences between the functional state of human T cells in mice created using these two different methods of creating scid mice/human chimeras presumably reflects the different natural histories of T cells that develop within a thymus xenograft in a scid mouse compared with postthymic T cells injected into a scid mouse.

Taken together, these data support the affinity model for intrathymic T-cell maturation. Once the TCR is expressed on the cell surface, the binding of antigen to the TCR may result in either positive selection or clonal deletion. When thymocytes expressing high density of TCR bind to an antigen on an APC with high affinity, cell death occurs due to clonal deletion.34 On the other hand, it has been postulated that a similar type of low-affinity interaction, involving fewer numbers of TCR bound to antigen molecules on APC, may result in positive selection.46 SE presented to CD4-8+ (double-positive) cells expressing the cognate TCR would be expected to be mitogenic, because of the small numbers of cognate TCR expressed on the surface that are available for SE binding. As double-positive cells begin to differentiate and express larger amounts of TCR, the effect of SE binding would vary according to the pathway along which they had differentiated. We see different effects of the intrathymic presentation of SE to CD4-8- and CD4-8+ cells expressing the cognate TCR, perhaps because SE has a specific affinity for class II HLA molecules,29,30,46,47 and the affinity of T cells expressing the CD4 coreceptor/TCR complex to SE in the presence of class II HLA on APC would be higher than the affinity of T cells expressing only the CD8 coreceptor/TCR complex to SE on APC.48 In both the CD4 and CD8 pathways, the single-positive, TCRαβ cells would be deleted, either due to the larger number of surface TCR engaged and/or loss of immediate progenitors. Based on this model, we would predict that negative intrathymic selection occurs late in intrathymic development, at a time when thymocytes have expressed high levels of TCR; positive selection occurs at earlier stages of T-cell development associated with lower levels of surface TCR.4 The availability of an in vivo model for human T-cell development will allow direct testing of some aspects of this model, and a direct comparison between the events of T-cell development occurring in mice (and other animals) and in humans.

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