Autoimmune Disease as a Consequence of Developmental Abnormality of a T Cell Subpopulation

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Summary

Neonatal thymectomy (NTx), especially around day 3 after birth, causes various organ-specific autoimmune diseases in mice. This report shows that: (*a*) T cells expressing the interleukin 2 receptor α chains (CD25) ontogenically begin to appear in the normal periphery immediately after day 3, rapidly increasing within 2 wk to nearly adult levels (~10% of $CD3^+$ cells, especially of $CD4^+$ cells); (*b*) NTx on day 3 eliminates $CD25^+$ T cells from the periphery for several days; inoculation immediately after NTx of $CD25^+$ splenic T cells from syngeneic non-Tx adult mice prevents autoimmune development, whereas inoculation of $CD25^-$ T cells even at a larger dose does not; and furthermore, (*c*) similar autoimmune diseases can be produced in adult athymic *nu/nu* mice by inoculating either spleen cell suspensions from 3-d-old euthymic *nu/+* mice or $CD25^+$ cell–depleted spleen cell suspensions from older, even 1-yr-old, *nu/+* mice. The $CD25^-$ populations from neonates or adults are also similar in the profile of cytokine formation. These results, taken together, indicate that one aspect of peripheral self-tolerance is maintained by $CD25^+$ T cells that sustain potentially pathogenic self-reactive T cells in a $CD25^-$ dormant state; the thymic production of the former is developmentally programmed to begin on day 3 after birth in mice. Thus, NTx on day 3 can, at least transiently, eliminate/reduce the autoimmune-preventive $CD25^+$ T cells, thereby leading to activation of the self-reactive T cells that have been produced before NTx.

Although the etiology of spontaneous autoimmune diseases is largely unknown at present, autoimmune disease may develop as a consequence of altered control of potentially pathogenic self-reactive lymphocytes that recognize antigenically normal self-constituents (1). Indeed, simple manipulation of the thymus/T cells, without exogenous immunization with self-antigens in potent adjuvant, can cause autoimmune disease in normal animals (for reviews see references 2 and 3). For example, neonatal thymectomy (NTx)[1], especially between days 2 and 4 after birth, of selected strains of normal mice, produces various autoimmune diseases, such as hemolytic anemia, thyroiditis, gastritis, oophoritis, or orchitis (4–11). In this report, we investigate the mechanism by which NTx causes autoimmune disease in normal mice, and thereby attempt to show how the normal immune system developmentally controls potentially pathogenic self-reactive T cells.

Based on various T cell abnormalities in NTx mice, several possible mechanisms have been proposed on the NTx-induced autoimmune disease, such as: insufficient clonal deletion in neonatal period and the inability of NTx mice to eliminate leaked self-reactive T cell clones that would be deleted in normal non-Tx mice upon recirculation through the thymus (12–15); reduction/elimination of an immunoregulatory T cell population by NTx (16); NTx-induced alteration of lymphocyte population kinetics towards expansion of self-reactive T cells (17, 18); or infection with autoimmune-eliciting viruses due to NTx-induced immunodeficiency (19). It remains obscure, however, which of these plausible mechanisms is the primary one by which NTx triggers autoimmune disease. To determine a particular NTx-induced T cell abnormality as the primary triggering event of the autoimmune development, correction of

[1]*Abbreviations used in this paper:* as, antisense; NTx, neonatal thymectomy; RT, reverse transcriptase, s, sense; Tx, thymectomy.

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the abnormality must prevent autoimmune disease in NTx mice, and, further, the same autoimmune disease must be produced by introducing the abnormality itself into normal mice directly, not indirectly by NTx.

We and others (20–23) have previously shown that autoimmune diseases, including those produced by NTx, spontaneously developed in normal rodents when $CD5^{high}$ or $CD45RB/C^{low}$ cells were eliminated from the peripheral $CD4^+$ population; reconstitution of the respective eliminated population prevented autoimmune development. Assuming that $CD4^+$ T cells capable of preventing NTx-autoimmune disease may well be $CD5^{high}$ and $CD45RB^{low}$ and begin to appear in the periphery around day 3 after birth in normal ontogeny, we have searched for a T cell surface molecule that is more specific than CD5 or CD45RB/C in delineating such an autoimmune-preventive $CD4^+$ population. This search revealed the IL-2 receptor α chain (CD25) molecule as a candidate, since CD25 is expressed on a subpopulation of $CD5^{high}$ and $CD45RB^{low}$ $CD4^+$ cells (24). In this report, we show that CD25-expressing T cells ontogenically begin to appear in the periphery immediately after day 3, and NTx-induced autoimmune disease can be prevented by inoculating $CD25^+$ splenic T cells from non-Tx normal mice; furthermore, similar autoimmune diseases can be produced in adult mice by directly eliminating peripheral $CD25^+$ T cells. Our results indicate that self-tolerance is developmentally established not only by clonal deletion but also by T cell–mediated control of self-reactive T cells.

Materials and Methods

Mice. BALB/c mice, BALB/c *nu/nu* mice of 6 wk of age, and BALB/c *nu/+* mice of 8 wk of age were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were maintained in the animal facility of the Institute of Physical and Chemical Research (RIKEN). To obtain newborn BALB/c mice, mice were mated in our facility.

NTx. Tx was performed on day 3 after birth (the day of birth as day 0). Mice were anesthesized by keeping them in crushed ice for 2–3 min, and the thymus was removed en bloc under a dissecting microscope with a cottonhead toothpick (25). Sham Tx was performed by cutting the sternum without removal of the thymus. The wound was sutured and the mice were kept at 30°C overnight and then returned to their mothers.

Preparation of T Cell Subpopulations. Lymphocyte suspensions (5×10^7) prepared from spleens and lymph nodes (inguinal, axillary, brachial, and mesenteric) were incubated in 12 × 75–mm glass tubes (Corning, Corning, NY) with 100 μl of 1:10-diluted ascites of anti-CD25 (7D4, rat IgM) (26), anti-L3T4 (CD4) (GK1.5, rat IgG2b) (27), or anti-Lyt-2.2 (CD8) (mouse IgG2a) (28) for 45 min on ice. They were washed once with HBSS (GIBCO BRL, Gaithersburg, MD) and incubated with 1.0 ml of nontoxic rabbit serum (GIBCO BRL) 1:5-diluted with Medium 199 (GIBCO BRL) for 30 min in a 37°C water bath with occasional vigorous shakings; 100 μg of DNase I (Sigma Chemical Co., St. Louis, MO) was added for the last 5 min of the incubation. The suspensions were then washed twice with HBSS, and injected intraperitoneally into NTx mice 1 wk after NTx or intravenously into 6–8-wk-old female *nu/nu* mice (24). To remove $CD4^+$ cells completely after anti-L3T4 + C treatment, the treated cells were incubated for 1 h at 4°C on plastic dishes precoated overnight with affinity-purified anti–rat IgG (Cappel-Organon Teknika, West Chester, PA), and nonadherent cells were collected (24). Less than 1% of lymphocytes after the anti-Lyt-2.2 + C, anti-L3T4 + C and subsequent panning treatment, or anti-CD25 (7D4) and anti-Lyt-2.2 + C treatment, were positive for FITC-rat anti-Lyt-2 (CD8) (53-6.7) (29), FITC-$F(ab')_2$ anti–rat IgG (Jackson ImmunoResearch, West Grove, PA), or anti-CD25 (PC61) (30) plus FITC-anti–rat IgG staining, respectively.

For enrichment of $CD25^+$ T cells, spleen and lymph node cell suspensions were first enriched for $CD4^+$ cells by incubating the cells with the mixture of culture supernatants of J11D (rat IgM) (31) and anti-CD8 (rat IgG) (29), and then panned on plastic dishes precoated overnight with affinity-purified anti–rat IgG (Cappel-Organon Teknika). The $CD4^+$ cells (>90% pure) were then panned on plastic dishes precoated overnight with purified 7D4 mAb (100 μg/ml), and the adherent cells were collected by scraping with a rubber policeman. They were 50–60% pure for $CD25^+$ cells. To obtain a pure $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ population for reverse transcriptase (RT)-PCR analysis (see below), cells stained with PE-anti-CD4 and FITC-anti-CD25 were sorted by a FACStar® flow cytometer with Consort 30 program (Becton Dickinson & Co., Mountain View, CA) as previously described (32).

Flow Cytometric Analysis. 10^6 cells were stained with FITC-anti-CD3 (145-2C11) (33) and biotinylated anti-CD25 (7D4), purchased from PharMingen (San Diego, CA), and PE-streptavidin (Biomeda, Foster City, CA) as the secondary reagent, and then analyzed by a FACScan® flow cytometer (Becton Dickinson & Co.), as previously described (24).

ELISA for Detecting Serum Autoantibodies. A detailed description of the ELISA (using alkaline phosphatase–conjugated secondary antibody and *p*-nitrophenyl disodium hexahydrate as the substrate) used to detect autoantibodies against gastric parietal cells was given earlier (34).

Histology and Criteria for Grading Autoimmune Disease. Tissues and organs (thyroid, lung, pancreas, stomach, adrenal gland, kidney, ovaries, or testes) were fixed in 10% formalin and processed for hematoxylin and eosin staining. Gastritis was graded from 0 to 2+ depending on macroscopic and histological severity: 0 = the gastric mucosa was histologically intact; 1+ = gastritis with histologically evident destruction of parietal cells and cellular infiltration of the gastric mucosa; and 2+ = severe destruction of the gastric mucosa accompanying the formation of giant rugae due to compensatory hyperplasia of mucous-secreting cells (see reference 20 for the giant rugae) (24).

RT-PCR. Total cellular RNA was isolated from 10^5 FACS®-sorted cells by modification of the guanidium isothiocyanate method (35). RNA was converted to cDNA with oligo-dT primer and Moloney murine leukemia virus RT (Toyobo, Osaka, Japan). The cDNA was preamplified with random 15-nucleotide primers; each aliquoted cDNA sample was then amplified with specific primers (see below) in Falcon Microtest III flexible U-bottomed, 96-well assay plates (Becton Dickinson Labware, Oxnard, CA) on a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA). PCR conditions were strictly defined for each primer pair. The following sequences (sense [s] and antisense [as]) were synthesized and used as the primers: HPRT (s) 5′-GATACAGGCCAGACTTGTTGG-3′, (as) 5′-GAGGGTAGGCTGGCCTATAGG-3′; CD4 (s) 5′-AGGTCAAGATGGACTCCAGG-3′, (as) 5′-GGCTCTTCTGCATCCGGTGG-3′; CD25 (s) 5′-CAGACATGCAGAAGCCAACAC-3′, (as) 5′-GGTGAGCCCGCTCAGGAGGA-3′; IL-2, (s)

5′-AAGATGAACTTGGACCTCTGCGG-3′, (as) 5′-CCTTATGTGTTGTAAGCAGGAGG-3′; IL-4 (s) 5′-ATGGGTCTCAACCCCCAGCTAGT-3′, (as) 5′-GCTCTTTAGGCTTTCCAGGAAGTC-3′; IL-10 (s) 5′-TCAAACAAAGGACCAGCTGGACAACATACTG-3′, (as) 5′-CTGTCTAGGTCCTGGAGTCCAGCAGACTCA-3′; IFN-γ (s) 5′-CATGAAAATCCTGCAGAGCCAG-3′, (as) 5′-TGCTGGCAGAATTATTCTTATTGG-3′; TGF-β (s) 5′-CTCCCACTCCCGTGGCTTCTAG-3′, (as) 5′-GTTCCACATGTTGCTCCACACTTG-3′; and TNF-α (s) 5′-CCACGTCGTAGCAAACCACC-3′, (as) 5′-AAGTACTTGGGCAGATTGACCTC-3′. The RT-PCR products were electrophoresed on 1.5% agarose (FMC BioProducts, Rockland, ME) gels and stained with ethidium bromide.

Results

Induction of Autoimmune Disease by NTx. Tx on day 3 after birth produced, in 3 mo, histologically evident gastritis accompanying high titers of circulating antiparietal cell autoantibodies in ~50% of BALB/c mice, and oophoritis with antioocyte autoantibodies in 20% of mice. Tx on day 0 or 7 was far less efficient for autoimmune induction (15% incidence of gastritis and no oophoritis), and Tx on day 14 or adult (6 wk of age) was unable to elicit autoimmunity (Fig. 1 *A*). Percent composition of $CD3^+$ cells in the lymph nodes of day 0, 3, or 7, or non-Tx mice at 3 mo of age was 17.1 ± 2.7, 23.1 ± 1.4, 45.0 ± 2.2, 69.0 ± 4.2, respectively (n = 5). NTx reduced a greater number of $CD4^+$ cells than $CD8^+$ cells: percent composition of $CD4^+$ or $CD8^+$ cells in the lymph nodes of day 3 Tx mice (n = 5) was 18.5 ± 7.5% and 9.5 ± 3.6%, respectively, compared with 44.0 ± 8.7% and 13.1 ± 0.6%, respectively, in sham Tx mice (n = 5).

The time course study of serological and histological development of gastric autoimmunity after day 3 Tx showed that significant titers of antiparietal cell autoantibodies became detectable by ELISA from 3 to 4 wk after Tx (Fig. 1 *B*). It took 5–6 wk for inflammatory destruction of the parietal cells to become histologically evident, although cellular infiltration to the gastric mucosa began earlier (data not shown). The degree of histological severity of the gastritis was well correlated with the titers of antiparietal cell autoantibodies when Tx mice were examined at 2 mo of age. These results indicate that pathogenic self-reactive T cells are activated within a couple of weeks after day 3 Tx.

Ontogeny of $CD25^+$ T Cells and Effects of NTx on the Ontogeny. By flow cytometric analysis, few splenic $CD3^+$ cells expressed CD25 in 3-d-old mice (0.06 ± 0.1%, n = 7); in contrast, a significant fraction of $CD3^+$ cells expressed CD25 in 7-d-old (5.48 ± 0.93%, n = 5) or adult mice (10.8 ± 1.01%, n = 11) (Fig. 2 *A*). In infant as well as adult mice, 5–10% of $CD4^+$ cells and <1% of $CD8^+$ spleen cells expressed CD25; the majority of $CD25^+$ cells did not coexpress IL-2Rβ (24). We noted that a larger fraction of B cells of neonatal mice expressed CD25, compared with adult mice (Fig. 2 *A*). These B cells may be immature B cells as demonstrated for a similar immature population in the adult bone marrow (36).

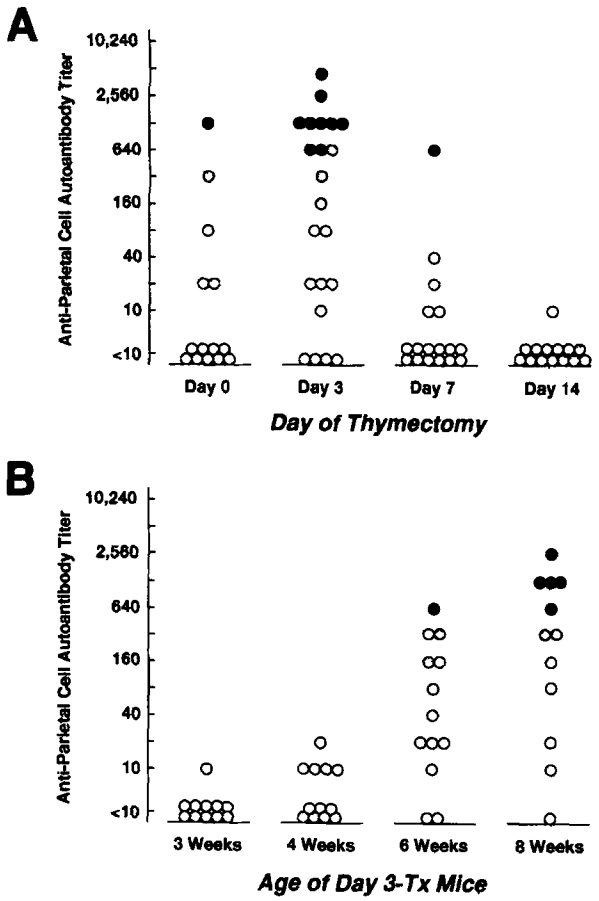

Figure 1. (*A*) Incidence of autoimmune gastritis in mice thymectomized on various days after birth. BALB/c mice received Tx on days 0 (within 24 h after birth), 3, 7, or 14. Mice were histologically and serologically examined 3 mo after Tx. (*B*) BALB/c mice were thymectomized on day 3 and examined for histological development of gastritis and titers of antiparietal cell autoantibodies at various ages as shown (●) Grade 2 gastritis; (○) grade 1 gastritis; and (○) intact gastric mucosa.

Similar analyses of CD25-expressing T cells in normal BALB/c mice at various ages revealed that $CD3^+$ $CD25^+$ cells began to appear in the spleen immediately after day 3, rapidly increasing within 2 wk to adult levels in percentage among $CD3^+$ cells (Fig. 2 *B*) and in absolute numbers (Fig. 2 *C*). Other mouse strains, such as A/J and C57BL/6 (which are high- and low-incidence strain, respectively, of NTx-induced autoimmune disease [9]), also showed ontogenic patterns of $CD25^+$ T cells similar to BALB/c (data not shown). Tx on day 3 not only reduced the number of $CD3^+$ cells, especially $CD4^+$ cells, but also depleted $CD3^+$ $CD25^+$ cells for 1 wk after Tx (Fig. 2 *C*). 3 mo after NTx, $CD25^+$ T cells constituted 19.6 ± 2.6% (n = 5) of splenic $CD4^+$ cells and 4.4 ± 2.6% (n = 5) of $CD8^+$ cells, indicating that they might have developed from the $CD25^-$ T cell population that had migrated to the periphery before NTx (see also Fig. 4).

Prevention of NTx-induced Autoimmune Disease by Inoculating $CD25^+$ T Cells from Syngeneic Normal Adult Mice. To determine whether $CD25^+$ $CD4^+$ cells prevent autoimmune

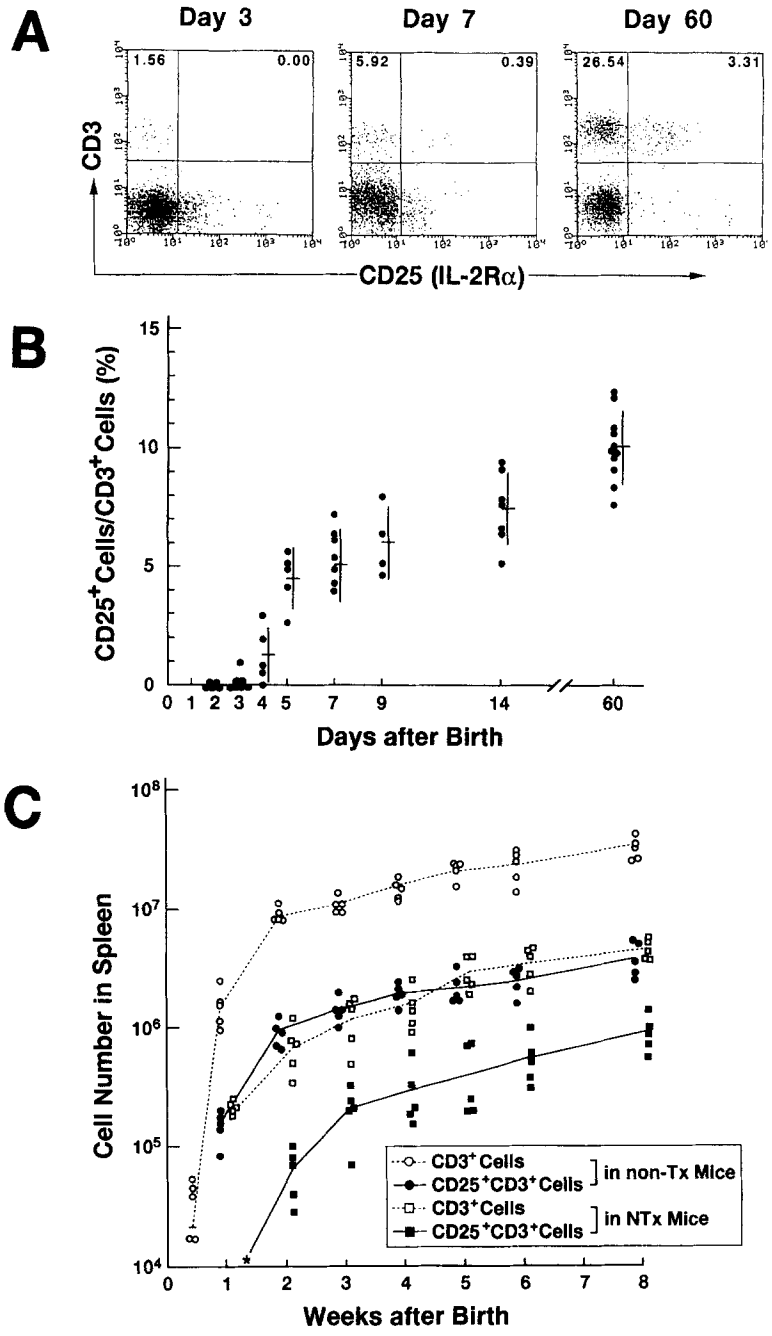

Figure 2. (*A*) Expression of CD25 on splenic $CD3^+$ cells from 3-, 7-, or 60-d-old BALB/c mice. Cells were stained with PE-anti-CD25 (*abscissa*) and FITC-anti-CD3 (*ordinate*). A representative staining of seven independent experiments is shown. (*B*) Percentages of $CD25^+$ cells among $CD3^+$ cells, calculated from CD3-CD25 staining as shown in *A*, in spleens from mice at various ages. (*Vertical bars*) Mean SD. (*C*) The number of $CD3^+$ and $CD25^+$ $CD3^+$ cells in spleens of day 3 Tx or non-Tx mice at various ages. The numbers were calculated from the total number of spleen cells and the percentages of each population in spleens, as shown in *A*. The number of $CD3^+$ $CD25^+$ cells in the spleens 1 wk after day 3 Tx was $<10^4$ per spleen (*asterisk*).

development in NTx mice, graded numbers of splenic T cell subpopulations from syngeneic non-Tx adult mice were inoculated into NTx mice 1 wk after NTx; the recipients were histologically and serologically examined 3 mo later (Fig. 3). $CD4^+$ $CD25^-$ cell suspensions, adjusted to an autoimmune-preventive dose of $CD4^+$ cells (10^7), failed to prevent gastritis. On the other hand, a small dose (2×10^6) of cell suspensions enriched for $CD4^+$ $CD25^+$ cells prevented autoimmunity, but an equivalent dose of $CD4^+$ cells did not. $CD8^+$ cells, ~1% of which expressed CD25 (24), were far less effective for prevention when compared with an equivalent dose of $CD4^+$ cells (10^7). We obtained similar results of $CD25^+$ cell–mediated autoimmune prevention on other NTx-induced autoimmune diseases in other strains, e.g., autoimmune oophoritis in A/J mice (15) (Sakaguchi, S., unpublished data). Taken together, these results indicated that the autoimmune-preventive activity was mainly, if not solely, in the $CD25^+$ $CD4^+$ population.

To determine the fate and CD25-phenotypic change of the inoculated CD4 subpopulations ($CD4^+$, $CD4^+$ $CD25^-$, or $CD4^+$ $CD25^+$ cells), they were prepared from the spleens of BALB/c *Thy-1*^{a} mice and inoculated into day 3 Tx BALB/c mice, which are *Thy-1*^{b} (Fig. 4). Higher numbers of $CD25^+$ Thy-1.1^+ T cells (~40% of Thy-1.1^+ cells)

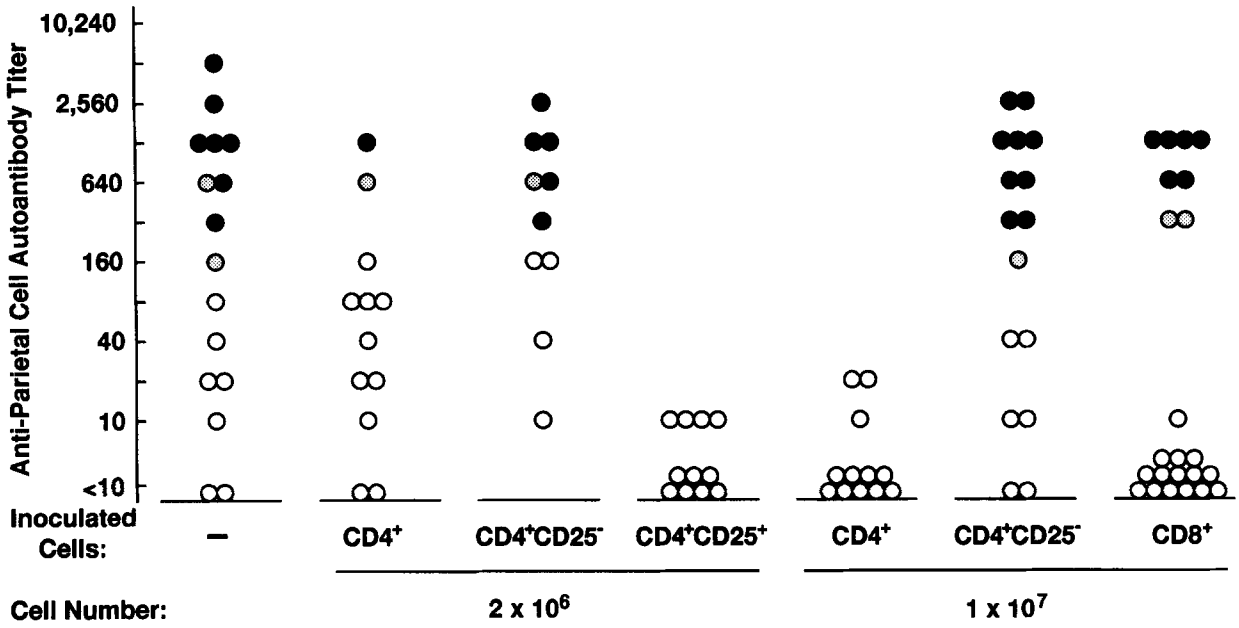

Figure 3. Graded numbers of lymphocyte suspensions as shown were inoculated into day 3 Tx BALB/c mice 1 wk after Tx. Mice were examined 3 mo later for histological and serological development of gastritis. (●, ○, ○) See legend to Fig. 1.

were present 2 mo after inoculation in the NTx mice inoculated with Thy-1.1$^+$ CD4$^+$ CD25$^+$ cell–enriched suspensions (50–60% pure for CD25$^+$ cells) compared with those inoculated with whole CD4$^+$ or CD4$^+$ CD25$^-$ cells (in these mice, CD25$^+$ cells constituted ~10% of Thy-1.1$^+$ cells). Thus, at least some of the inoculated CD25$^+$ T cells persisted and presumably proliferated as CD25$^+$. Furthermore, appearance of CD25$^+$ Thy-1.1$^+$ cells in NTx mice inoculated with CD25$^-$ Thy-1.1$^+$ cells indicated that a fraction of inoculated CD25$^-$ cells had differentiated to CD25$^+$ cells, as was also suggested by the finding that NTx mice developed CD25$^+$ T cells (Fig. 2 *C*).

Induction of Autoimmune Disease in nu/nu Mice by Transfer of Newborn Spleen Cells or CD25$^+$ Cell–depleted Spleen Cells from Older Mice at Any Age. The above results indicate that the peripheral T cell population of 3-d-old mice may be deficient in autoimmune-preventive CD25$^+$ T cells but may contain some self-reactive T cells, and mice older than 3 d of age may bear pathogenic self-reactive T cells and sufficient numbers of CD25$^+$ autoimmune-preventive T cells to control them. To demonstrate this, we inoculated splenic cell suspensions, prepared from BALB/c *nu/+* mice at various ages, into adult BALB/c *nu/nu* mice that were histologically and serologically examined 3 mo later for the development of autoimmune disease (Table 1). The *nu/nu* mice transferred with spleen cells from 3-d-old *nu/+* mice developed autoimmune diseases, including gastritis and oophoritis, whereas those transferred with spleen cells from 14-d or 8-wk-old *nu/+* mice developed few or no autoimmunities. In contrast, when the inocula from adult mice were depleted of CD25$^+$ cells by treatment with anti-CD25 and C, the recipients developed autoimmune diseases. The spectrum of affected organs and incidences of the autoimmune diseases were dependent on the dosages of inoculated CD25$^-$ cells.

Inoculation of spleen cells from 3-d-old *nu/+* mice into 3-d-old *nu/nu* littermates produced autoimmune disease as well. This indicates that the condition of T cells in 3-d-old mice may be critical for autoimmune induction, whereas age-dependent differences, if any, in the conditions of the target antigens (37), the functions of antigen-presenting or B cells (38, 39), or other host conditions, may not.

Furthermore, comparable incidences of autoimmune diseases developed in *nu/nu* mice whether the transferred CD25$^-$ cells were prepared from 2 or 12-mo-old *nu/+* mice. Thus, the potentially pathogenic self-reactive T cells were present in the periphery of aged as well as neonatal or young mice; they were not significantly deleted (12–15, 40), rendered anergic (14), or diluted out in the periphery with aging (13). Moreover, elimination/reduction of CD25$^+$ T cells could activate the self-reactive T cells at any age.

Transcription of Cytokine Genes in Splenic CD4$^+$ Cells from Mice at Various Ages. To characterize further the autoimmune-inducing or autoimmune-preventive CD4$^+$ cells, we analyzed transcription of cytokine genes (IL-2, IL-4, IL-10, IFN-γ, TNF-α, and TGF-β) in CD4$^+$ cell suspensions (10^5) prepared by cytofluorometric cell sorting from the spleens of 3-, 7-, or 60-d-old BALB/c mice, or the same number of CD4$^+$ CD25$^+$ or CD4$^+$ CD25$^-$ splenic cells from 60-d-old BALB/c mice. This RT-PCR method with amplification of cDNAs by random primers is sufficiently sensitive to detect a single lymphocyte quantity of cytokine mRNA (Toda, M., and S. Sakaguchi, manuscript in preparation). The analysis shown in Fig. 5 confirmed the result

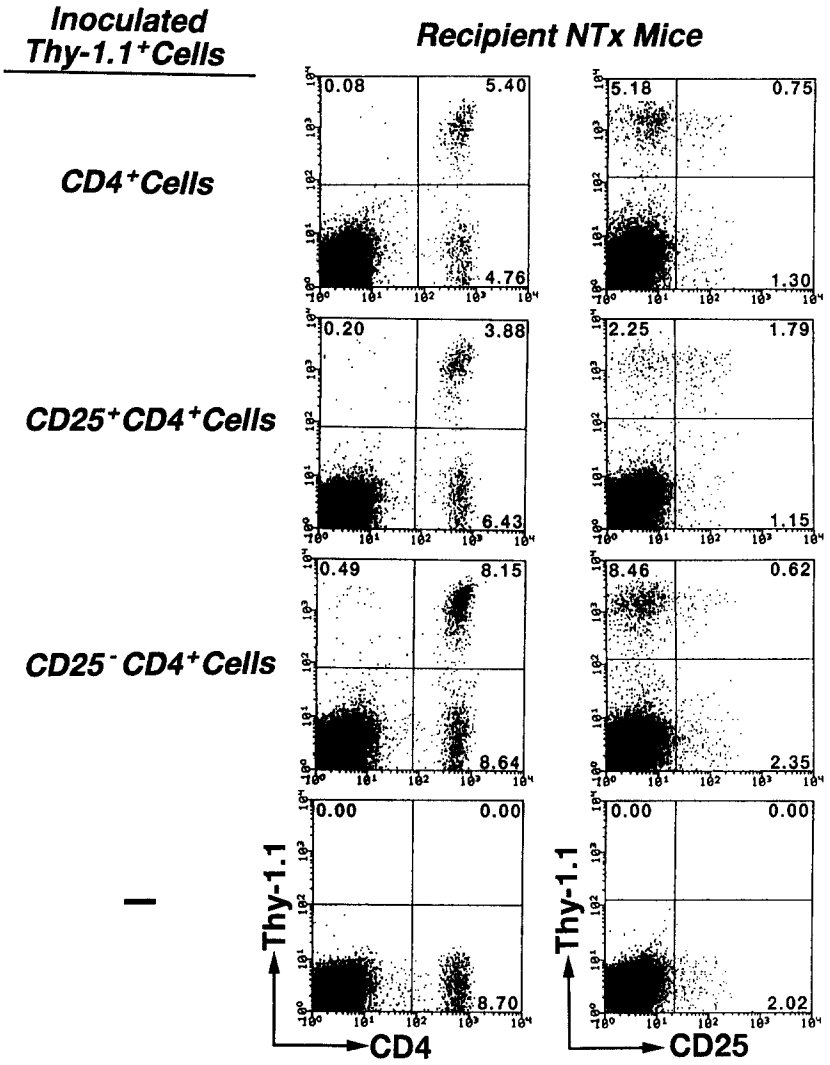

Figure 4. Day 3 Tx BALB/c mice were inoculated with 2×10^6 $CD4^+$, $CD4^+$ $CD25^-$, or $CD4^+$ $CD25^+$ splenic cells prepared from Thy-1.1 BALB/c congenic mice and examined 2 mo later. Lymph node cells were stained with FITC-anti-Thy-1.1 (*ordinate*) and PE-anti-CD4 or PE-anti-CD25 (*abscissa*). A representative staining of four independent experiments is shown.

of cytofluorometric study that few $CD4^+$ cells of 3-d-old mice express CD25 (Fig. 2 *A*). $CD4^+$ cells from 3-, 7-, or 60-d-old mice, as well as $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ cells from 60-d-old mice, transcribed a comparable level of IL-2, IFN-γ, or TNF-α gene. In contrast, IL-4, IL-10, or TGF-β transcripts were predominantly detected in $CD4^+$ cells from 7-d-old or adult mice, especially in $CD25^+$ $CD4^+$ cells, compared with $CD4^+$ cells from 3-d-old mice or $CD25^-$ $CD4^+$ cells from adults. Thus, the $CD4^+$ cells of 3-d-old mice were similar to $CD25^-$ $CD4^+$ cells of adult mice not only in the nonexpression of CD25 molecules but also in their cytokine profile; the $CD25^+$ $CD4^+$ population, which began to appear in the periphery immediately after day 3, predominantly contained cells forming IL-4, IL-10, and/or TGF-β.

Discussion

The main conclusion from the data in this report is that one aspect of peripheral self-tolerance is maintained by $CD25^+$ T cells that sustain potentially pathogenic self-reactive T cells in a $CD25^-$ dormant state. The ontogenic time course of the former is intrinsically programmed as the developmental process in normal mice and abnormality in this process can cause autoimmune disease in genetically susceptible individuals.

We showed that $CD25^+$ T cells ontogenically began to appear in the periphery of normal mice from day 3 after birth. Hence, Tx on day 3 transiently depleted/reduced $CD25^+$ T cells from the periphery, inoculation of $CD25^+$ T cells from non-Tx mice effectively prevented autoimmune development, and transfer of $CD25^-$ T cells from 3-d-old or older *nu/+* mice successfully induced similar autoimmune disease in *nu/nu* mice. These results, taken together, indicate the following: (*a*) the thymus of an apparently nonautoimmune strain of mice (such as BALB/c) produces not only potentially pathogenic self-reactive T cells (which persist in the normal periphery at a $CD25^-$ dormant state) but also $CD25^+$ T cells capable of downregulating the activation/expansion of self-reactive T cells

Table 1. *Induction of Autoimmune Disease in BALB/c nu/nu Mice by Inoculating T Cells from BALB/c nu/+ mice at Various Ages*

Exp. group	Age of donors	No. of cells treated	Treatment of cells	No. of mice	No. of mice with autoimmune disease		
					Gastritis	Oophoritis	Thyroiditis
A	3 d	1–2 × 10^7	−	7*	3 (42.9)	1 (14.3)	0
B	3 d	3 × 10^7	−	12	6 (50.0)	5 (41.7)	0
C	14 d	3 × 10^7	Anti-CD25 + C	8	8 (100)	5 (62.5)	0
D	14 d	3 × 10^7	C	8	1 (12.5)	0	0
E	8 wk	3 × 10^7	Anti-CD25 + C	8	8 (100)	6 (75.0)	3 (37.5)
F	8 wk	3 × 10^7	C	12	0	0	0
G	8 wk	2 × 10^6	Anti-CD25 + C	8	4 (50.0)	2 (25.0)	0
H	8 wk	2 × 10^6	C	8	0	0	0
I	1 yr	3 × 10^7	Anti-CD25 + C	8	7 (87.5)	4 (50.0)	0

Indicated numbers of spleen cells from 3-d-old female BALB/c *nu/+* or +/+ mice were inoculated into 3-d-old littermate *nu/nu* mice (*) (group *A*) or 6-wk-old BALB/c *nu/nu* mice (group *B*). In groups *C–I*, indicated numbers of spleen cells from *nu/+* or +/+ mice at various ages were treated with anti-CD25 and complement (*C*), or C alone, and then inoculated into 6-wk-old female BALB/c *nu/nu* mice. The recipient *nu/nu* mice were histologically examined 2 mo later. Number of mice bearing respective autoimmune diseases (with histological severity of grade 1 and 2, see Materials and Methods and references 20, 24) is shown with percent incidence in parentheses. Note that $CD25^-$ cell suspensions prepared from 2 × 10^6 adult *nu/+* spleen cells (group *G*) and from 3 × 10^7 3-d-old nu/+ spleen cells (group *B*) contained an equivalent number of $CD25^-$ T cells (see Fig. 2 A).

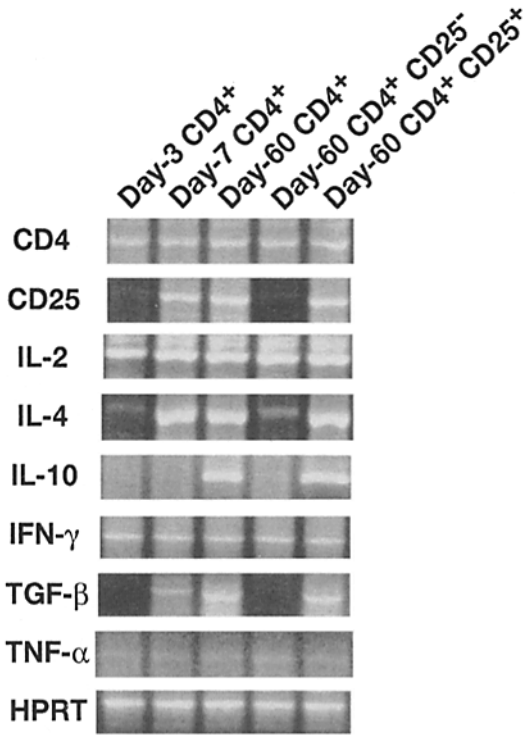

Figure 5. Transcriptions of various cytokine mRNAs were assessed by RT-PCR with the same number (10^5) of $CD4^+$ cells sorted by FACS® from spleens of 3-, 7-, or 60-d-old BALB/c mice. $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^+$ spleen cells (10^5) prepared from 60-d-old BALB/c mice by FACS® were also examined.

from the $CD25^-$ dormant state; (*b*) the production of the former begins before day 3, whereas production of the latter immediately after day 3; hence, (*c*) NTx on day 3 may abrogate, at least transiently, the $CD25^+$ cell–mediated control on the self-reactive T cells that have been produced before day 3, allowing some of them to become activated, expand, and eventually cause clinically and histologically evident autoimmune diseases. Judging from the rapid increase of $CD25^+$ T cells from day 3 on, it is likely that mice at 7 d of age have already harbored sufficient numbers of $CD25^+$ regulatory cells to control $CD25^-$ self-reactive T cells; on the other hand, 0-d-old mice, as deficient of the $CD25^+$ population as 3-d-old mice, bear much smaller numbers of $CD25^-$ self-reactive T cells than 3-d-old mice. Hence, both day 0 and day 7 Tx are far less efficient for autoimmune induction than day 3 Tx.

One may argue, however, that a quantitative change of T cell number in ontogeny, not a qualitative change of T cell subpopulations, may be responsible for autoimmune development after NTx. For example, a T-lymphocytopenia due to NTx might incur viral infection and the resultant activation of antigen-presenting cells might lead to activation of self-reactive T cells (19). This does not seem to be the case for the following reasons. First, a deliberate preparation of T-lymphocytopenia in *nu/nu* mice by inoculating a small number (e.g., 2 × 10^6) of *nu/+* spleen cells failed to produce autoimmune disease, whereas a large number of $CD25^-$ *nu/+* T cells (e.g., prepared from 3 × 10^7 spleen cells) effectively produced autoimmune disease. Second, transplantation of *nu/+* newborn thymuses in *nu/nu* mice produced similar autoimmune diseases without T-lymphocytopenia (41). Third, others have shown that mainte-

nance of germ-free NTx mice in germ-free conditions could not prevent autoimmune development (42).

The autoimmune-preventive $CD25^+$ $CD4^+$ cells in normal unimmunized mice were apparently indistinguishable from "activated," "memory," or "effector" T cells not only in their expression patterns of various cell surface molecules (including CD45RB and CD5 [24]) but also cytokine formation (Fig. 5) (43–45). Indeed, the $CD25^-$ T cell population gave rise to $CD25^+$ activated, effector T cells (24, and Fig. 4). Furthermore, polyclonal $CD25^+$ activated T cell blasts prepared by mitogen stimulation of normal T cells effectively prevented NTx-induced autoimmune disease (Sakaguchi, S., unpublished data). Although it remains to be determined whether any T cells acquire the autoimmune-preventive activity upon activation, these findings suggest that the ontogenic time course and the basal number of the autoimmune-preventive $CD25^+$ population may be developmentally programmed, but the size of the population may not be innately fixed and may increase when T cells are activated in immune responses to non-self antigens (24). To elucidate further the mechanism of this $CD25^+$ T cell–mediated control, it is currently under investigation whether cytokines, such as IL-4, IL-10, or TGF-β, predominantly produced by $CD25^+$ $CD4^+$ T cells (Fig. 5), are responsible for sustaining self-reactive T cells in the $CD25^-$ dormant state.

The $CD25^+$ T cell–mediated immunoregulation is the key, but not the sole, mechanism of controlling pathogenic self-reactive T cells. Indeed, others have shown that intrathymic expression of the gastric parietal cell antigens inhibited NTx-induced gastritis (46, 47). Given the fact that T cells bearing high affinity TCRs for intrathymic self-antigens or peripheral organ–specific antigens circulating to the thymus can be clonally deleted (even at a much lower antigen concentration than that required for activation [48]), it is likely that potentially pathogenic self-reactive T cells that have escaped the thymic-negative selection, may bear low affinity TCRs for the relevant self-antigens, hence high thresholds for activation. The $CD25^+$ T cell–derived suppressive signal may further raise the thresholds, thereby stably maintaining them in the $CD25^-$ dormant state even when they recognize self-peptides presented by antigen-presenting cells (49). By contrast, some, if not all, T cells specific for non-self antigens may easily overwhelm the suppression and become activated upon antigen stimulation presumably because of their high affinity TCRs for the antigens, and hence, low activation thresholds, even though the $CD25^+$ T cell–derived suppressive signal may raise the thresholds to the same extent as for self-reactive T cells. The T cell–mediated peripheral immunoregulation and the thymic clonal deletion may thus cooperatively sustain stable self-tolerance while enabling effective immune responses to non-self antigens.

The present findings in mice may also apply to other species and other autoimmune diseases. For example, NTx causes or aggravates thyroiditis in chickens (50) and rats (51). Furthermore, spontaneous autoimmune diseases (e.g., diabetes mellitus and thyroiditis in NOD mice or BB rats) can be prevented by inoculating $CD4^+$ cells from the histocompatible normal animals before clinical onset of autoimmune disease (52, 53). The autoimmune-preventive $CD4^+$ T cells in these models might be $CD25^+$, and the models might have genetically determined abnormality in the $CD25^+$ cell–mediated control of self-reactive T cells. Further molecular analysis of the autoimmune-preventive activity of $CD25^+$ T cells and search for environmental agents or genetical abnormalities reducing $CD25^+$ T cells, retarding their ontogeny, or affecting their autoimmune-preventive activity, would contribute to our understanding of the etiology and pathogenetic mechanism of autoimmune disease.

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