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Epitope Spreading Is Not Required for Relapses in Experimental Autoimmune Encephalomyelitis[1]

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The sequential emergence of specific T lymphocyte-mediated immune reactivity directed against multiple distinct myelin epitopes (epitope spreading) has been associated with clinical relapses in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). Based on this association, an appealing and plausible model for immune-mediated progression of the advancing clinical course in MS and EAE has been proposed in which epitope spreading is the cause of clinical relapses in T cell-mediated CNS inflammatory diseases. However, the observed association between epitope spreading and disease progression is not universal, and absolute requirements for epitope spreading in progressive EAE have not been tested in the absence of multiple T cell specificities, because most prior studies have been conducted in immunocompetent mouse strains that possessed broad TCR repertoires. Consequently, the precise nature of a causal relationship between epitope spreading and disease progression remains uncertain. To determine whether relapsing or progressive EAE can occur in the absence of epitope spreading, we evaluated the course of disease in mice which possessed only a single myelin-specific TCR. These mice (transgenic/SCID +/+) exhibited a progressive and sometimes remitting/relapsing disease course in the absence of immune reactivity to multiple, spreading myelin epitopes. The results provide direct experimental evidence relevant to discussions on the mechanisms of disease progression in MS and EAE. ***The Journal of Immunology*****, 2003, 170: 1690–1698.**

Multiple sclerosis (MS)[3] is a debilitating paralytic autoimmune disease in which T lymphocyte-initiated CNS inflammation is thought to cause the crucial pathologic changes associated with clinical disability and paralysis (1). Despite significant advances in our understanding of T cell immunity and CNS inflammation, the basic etiology of MS remains uncertain and mechanisms initiating disease onset and progression are still not completely clear (2). Experimental autoimmune encephalomyelitis (EAE) has proven to be a particularly useful animal model in which to understand mechanisms of immune-mediated CNS pathology and the progressive clinical course (3). Convincing evidence in EAE has demonstrated that disease progression is often accompanied by the emergence of immune reactivity against an expanding array of myelin target epitopes (epitope spreading) (4) with a concomitant reduction in reactivity against the disease-inducing epitope (5). Epitope spreading in EAE has been observed to involve T cells specific for additional epitopes on the inducing myelin protein Ag (intramolecular spreading) (6), T cells specific for different myelin proteins (intermolecular spreading) (7), and, in F_1 hybrids, T cells restricted by a different H-2 allotype than that of the inducing T cells (8). Activation of naive T cells through endogenous self-priming to myelin Ags may occur in lymph nodes (9) and has been proposed as the disease-dependent mechanism responsible for epitope spreading (6).

In EAE models such as the SJL mouse where disease progression includes distinct phases of remission and relapse, epitope spreading has been associated with distinct periods of clinical worsening. Induction of specific tolerance to a spreading epitope induced both an inability to detect reactivity to the spreading epitope and an absence of relapse, suggesting that T cells specific for the spreading determinants were required for the relapsing clinical course (7, 10, 11). Epitope spreading, following a predictable sequence of emerging epitopes, also occurred during clinical progression in the absence of distinct episodes of clinical worsening or relapses in SWXJ F_1 mice (5, 12), again demonstrating a temporal association between epitope spreading and disease progression.

In contrast to the association between epitope spreading and disease progression observed in some studies, other studies did not find this association, and current models for epitope spreading do not appear to have incorporated the contrary results (13). Epitope spreading has been detected in (SJL $\times$ B10.PL)F_1 mice in the absence of clinical disease (14), suggesting that epitope spreading may be a disease-independent immune phenomenon which is not necessarily sufficient to cause significant pathology in the CNS. Detectable epitope spreading was absent following passive but not active relapsing disease induction in SJL mice (15), suggesting that epitope spreading depends on the experimental method of disease induction and is not a necessary component of disease progression. The predominance of cells specific for the immunizing epitope (proteolipid protein (PLP)$_{139-151}$) has been shown in the CNS through acute disease, remission, and relapse, suggesting that a persistent and focused response to the immunizing peptide rather than a spreading response to additional myelin epitopes was responsible for disease progression in SJL mice (16). Induction of remitting/relapsing EAE by the adoptive transfer of histocompatible, myelin basic protein (BP)-specific CXJ strain (H-2^d) T cell

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[3] Abbreviations used in this paper: MS, multiple sclerosis; BP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis; Tg, transgenic; PLP, proteolipid protein; gDNA, genomic DNA; LN, lymph node; Ac, acetylated.

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lines in SCID mice (H-2^d) demonstrated that clinical disease progression, including remission and relapse, did not require recipient-derived immune reactivity against multiple myelin epitopes (17). Thus, a substantial body of evidence suggests that epitope spreading is neither necessary nor sufficient to cause disease progression or relapses.

We have sought to evaluate requirements for epitope spreading during disease progression in the absence of immune reactivity against multiple epitopes. We have generated a spontaneous and adoptive mouse EAE model in which T cell immune reactivity is limited by expression of a single TCR specific for the myelin epitope $BP_{1–11}$ restricted by H-2^u. This was accomplished by using a breeding approach in which a mouse strain possessing an encephalitogenic transgenic (Tg) TCR specific for an epitope of BP ($BP_{1–11}$) restricted by H-2^u was crossed with the T cell-deficient C.B-17 *scid/scid* (designated as SCID) strain to generate a strain of H-2^u mice possessing exclusively the single encephalitogenic Tg TCR. Epitope spreading cannot occur in such mice due to the presence of only a single TCR and the lack of any additional T cell specificities. The course of paralytic disease in these animals demonstrated that disease progression and relapses occurred in the absence of epitope spreading.

Materials and Methods

Animals

B10.PL mice (H-2^u) expressing transgenic (Tg) TCR α- and β-chains specific for the acetylated (Ac) N-terminal residues 1–11 of BP restricted by I-A^u were provided by C. Janeway (Yale University, New Haven, CT). Generation of these mice was previously described (18). BP-specific Tg TCR B10.PL mice contain transgenes encoding the TCR α- (αV4) and β-chains (βV8.2) specific for the encephalitogenic epitope contained within $BP_{1–11}$. Lymphocyte-deficient C.B-17 *scid/scid* (designated as SCID) mice (19–21) were obtained from the SCID mouse colony (Veterinary Medical Unit, Veterans Affairs Medical Center, Portland, OR). Tg TCR mice possessing the SCID phenotype (Tg$^+$/SCID$^+$, +/+) were generated in our colony by first crossing BP-specific Tg TCR B10.PL (H-2^u) mice with immunodeficient C.B-17 *scid/scid* mice (H-2^d). F_1 offspring were crossed, and three strains of H-2^u mice were identified and propagated from the F_2 offspring: Tg TCR SCID mice (Tg$^+$/SCID$^+$, +/+); nontransgenic TCR SCID mice (Tg$^-$/SCID$^+$, −/+); and nontransgenic TCR, non-SCID mice (Tg$^-$/SCID$^-$, −/−). Mice in generations F_3–F_6 were used as study subjects in this report to evaluate the EAE disease course.

Previously, transfer of normal splenocytes was shown to protect immunodeficient Tg TCR mice from developing spontaneous EAE (22). To prevent Tg/SCID +/+ breeders from developing spontaneous EAE, each breeder was injected with spleen cells from one 8-wk-old Tg/SCID −/− donor. The spleen cells were prepared as a single-cell suspension. After spinning down the cells, the splenocytes were treated with RBC lysing buffer (1 ml/spleen; Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions, washed three times in ice-cold RPMI 1640, resuspended in 0.5 ml of RPMI 1640, and injected i.p. into each Tg/SCID +/+ recipient.

Phenotypic screening of Tg/SCID mice

For mAb staining, ~50 μl of blood of 5-wk-old mice was collected in 1 ml of PBS with EDTA (3 mg/ml). RBC were removed by hypotonic lysis. Cells were washed twice and resuspended in ~100 μl of staining medium/tube (3% FBS (Life Technologies, Grand Island, NY) and 0.02% sodium azide in sterile PBS). The anti-clonotypic monoclonal mouse Ab (3H12) specific for the mouse $BP_{1–11}$ TCR was kindly provided by J. Lafaille (Skirball Institute of Biomolecular Medicine and New York University Medical Center, New York, NY) (22). Two-color immunofluorescence using 3H12 (detected with a second Ab, anti-mIgM-FITC (BD PharMingen, San Diego, CA)) and anti-mCD4-PE (BD PharMingen) was performed on Ab-stained cells. Determination of H-2K^d expression was accomplished using a fluorescent mAb specific for mouse H-2K^d (BD PharMingen). Samples were analyzed on a FACScan with CellQuest software (BD Biosciences, Mountain View, CA).

PCR assay

To screen for the expression of the MHC haplotype H-2^u, genomic DNA (gDNA) was prepared for PCR from 2–3 mm of tail tissue by proteinase K digestion. PCR were performed using 1 μl of gDNA with a primer pair specific for H-2^u (sequences provided by J. LaFaille). Primers were prepared by the Molecular Microbiology and Immunology Laboratory Core Facility at Oregon Health & Science University. The PCR reagents used were obtained from Life Technologies, and PCR were performed on the GeneAmp PCR system 9600 (PerkinElmer, Wellesley, MA). The PCR conditions were as follows: one denaturation cycle of 94.5°C for 5 min followed by 26 cycles of 94.5°C for 30 s, 57°C for 60 s, and 72°C for 60 s, ending with a final extension at 72°C for 7 min.

Evaluation of clinical disease severity (paralysis)

Unimmunized and actively immunized Tg/SCID +/+ mice, and Tg/SCID −/+ and −/− mice that received an injection of encephalitogenic T cells were followed daily and scored for disease severity. Clinical disease was scored daily according to the following scale (0–9): 0, normal; 1, limp tail or mild hindlimb weakness; 2, limp tail and mild hindlimb weakness; 3, moderate hindlimb weakness or mild ataxia; 4, moderately severe hindlimb weakness; 5, severe hindlimb weakness; 6, paraplegia or moderate ataxia; 7, paraplegia and mild forelimb weakness; 8, paraplegia and moderate forelimb weakness; and 9, paraplegia and severe forelimb weakness or severe ataxia. Animals rarely developed ataxia in this model. Disease onset was defined as the first day on which clinical disease occurred. Recovery was defined as an improvement of two or more in disease score lasting at least 2 days. A relapse was defined as an increase of two or more in clinical score, lasting at least 2 days, after recovery. For Table IV only, an additional category of relapse was included in which there was an increase of two or more in clinical score, after a period of stable score lasting 5 or more days.

Peptides

Peptide for immunization ($BP_{1–11}$) and all other peptides were prepared on the 432A Peptide Synthesizer Synergy (Applied Biosystems, Foster City, CA). The peptide sequences were as follows: $BP_{1–11}$, ASQKRPSQRSK; $BP_{87–99}$, VHFFKNIVTPRTP; $PLP_{139–151}$, HSLGKWLGHPDKF; and $PLP_{178–191}$, NTWTTCQSIAFPSK (5, 23).

Immunization

Immunization for induction of active EAE or selection of encephalitogenic T cells was accomplished by s.c. injection with 0.2 ml (total/mouse) of CFA, an oil-in-water emulsion containing IFA, 200 μg of heat-killed *Mycobacterium tuberculosis* (strain H37Ra; Difco, Detroit, MI), and 150 μg of $BP_{1–11}$ peptide, injected into four sites.

Proliferation assay

The in vitro proliferative response during stimulation was assessed by [^{3}H]thymidine incorporation. In 96-well plates, either lymph node (LN) cells alone, splenocytes alone (4×10^5 cells/200 μl/well), or spleen-derived line cells (2×10^4 T cells/200 μl/well in the presence of irradiated thymic APC from Tg/SCID −/− donors (8×10^5 cells/200 μl/well)) were cultured for 2 days before adding [^{3}H]thymidine for the last 18 h of culture. Cells were cultured in triplicate wells in the absence or presence of 10 μg/well peptide Ag ($BP_{1–11}$, $BP_{87–99}$, $PLP_{139–151}$, $PLP_{178–191}$), 0.2 μg/well of the mitogen Con A (Sigma-Aldrich), 5 μg/well purified protein derivative of *M. tuberculosis* (Statens Serum Institut, Copenhagen, Denmark), or 100 U/well recombinant human IL-2 (Hoffmann-LaRoche, Nutley, NJ).

Adoptive cell transfer and selection of Tg TCR T cell lines

A T cell line was prepared from pooled immune spleens. Eleven days after immunization, draining LN and spleens from seven animals were collected in ice-cold sterile RPMI 1640. Single-cell suspensions of both LN and spleens were prepared by gently pressing the organs through sterile 200-mesh stainless steel screens. Splenocytes were treated with RBC lysing buffer. Cells were washed three times in ice-cold sterile RPMI by centrifugation at 1200 rpm for 10 min and resuspended in stimulation medium (10% FBS (Life Technologies), 100 μg/ml sodium pyruvate (Life Technologies), 2 mM L-glutamine (Life Technologies), and 5×10^{-5} M 2-ME in RPMI 1640 (Life Technologies)) and counted. Per petri dish, 50×10^6 splenocytes were stimulated with 25 μg of Con A in a total of 10 ml of stimulation medium. Cultured cells were incubated at 37°C and 7% CO_2. After 48 h of in vitro stimulation, spleen cells were harvested, washed three times in ice-cold RPMI 1640, and the viable, trypan blue-negative blasts were counted. Cells were transferred into naive recipients or were expanded for 5–7 days in IL-2-containing medium before restimulation with Con A (2.5 μg/ml) in the presence of irradiated histocompatible APCs (5×10^6 T cells and 80×10^6 APCs per 10 ml plate).

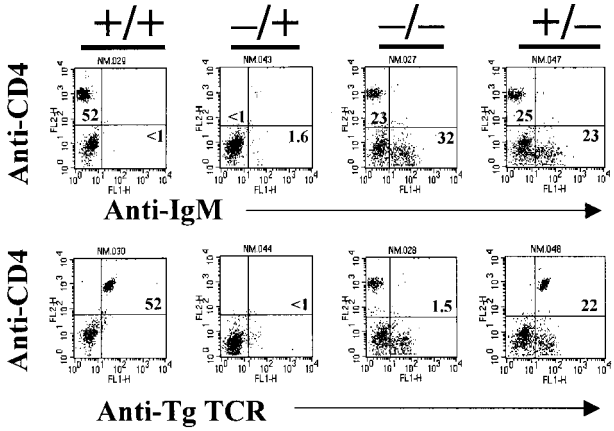

FIGURE 1. Representative dot plots of PBMC from single Tg/SCID mice with percentage of staining indicated (background isotype control subtracted) display unique mAb staining patterns that distinguish the various Tg/SCID strains. *Upper row* used anti-IgM-FITC vs anti-CD4-PE and *lower row* used anti-Tg TCR Ab (3H12; provided by J. LaFaille) with anti-IgM-FITC, second Ab vs anti-CD4-PE. +/+, Tg TCR$^+$/SCID$^+$; −/+, Tg TCR$^-$/SCID$^+$; −/−, Tg TCR$^-$/SCID$^-$; +/−, Tg TCR$^+$/SCID$^-$.

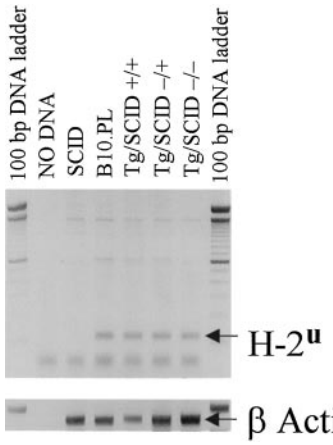

FIGURE 2. Detection of H-2^u-specific PCR amplification products from different donor sources of gDNA. Tg/SCID mice expressing H-2^u generated a specific band equivalent to that generated by B10.PL (H-2^u) donors. SCID mice (H-2^d) or Tg/SCID mice not expressing H-2^u did not generate the H-2^u-specific band and were not used in experiments or as breeders. β-actin-specific primer products were evaluated to control for absence of DNA in the assays.

For adoptive transfer after organ stimulation or restimulation, a portion of the blasts were resuspended at a concentration of 10–20 × 10^6 cells/0.5 ml of RPMI 1640 at room temperature, before transfer into recipient Tg/SCID mice (injection volume of 0.5 ml i.p.). The remainder of the cells were plated at a concentration of 20 × 10^6 cells/ml and expanded in growth medium (stimulation medium with IL-2). After 5 days of IL-2-mediated expansion, spleen cells (5 × 10^6 cells/plate) were restimulated with 25 μg of Con A in the presence of 80 × 10^6 irradiated thymocytes from Tg/SCID −/− donors in a total volume of 10 ml of 10% FBS stimulation medium.

Results

Development of Tg/TCR +/+ mice expressing H-2^u

The initial goal was to produce EAE-susceptible TCR Tg mice that lacked the capacity to rearrange the *TCR* genes necessary to generate additional myelin specificities. Tg TCR mice were first bred with SCID mice to produce F_1 offspring. F_1 mice were crossed to produce F_2 offspring, which were screened by fluorescent mAb staining and flow cytometry for the absence of surface IgM-positive B cells to identify mice expressing the SCID phenotype (homozygous for the *scid* mutation) and to distinguish these from non-SCID mice possessing surface IgM-positive B cells. F_2 mice were also screened for expression of the BP Ac1–11-specific Tg TCR using the anti-clonotypic 3H12 mAb, and for expression of CD4. Four distinct phenotypic expression patterns were identified (see examples, Fig. 1) and used to assign unique phenotypes to the four groups of mice (Table I). F_2 and subsequent generations of Tg/SCID +/+, −/+, and −/− mice identified in this way were inbred (sibling matings) to propagate the various strains of mice expressing these distinct Tg/SCID phenotypes (Tg TCR SCID mice (Tg/SCID +/+); nontransgenic TCR SCID mice (Tg/SCID −/+); and nontransgenic TCR, non-SCID mice (Tg/SCID −/−)). The Tg/SCID +/− strain was not propagated for this study, because these mice would be expected to possess all of the capacity for epitope spreading and disease progression as the nontransgenic immunocompetent B10.PL mice have.

F_2 and subsequent generations of offspring were also screened for expression of H-2^u, the restricting H-2 allotype for the Tg TCR. H-2^u-expressing Tg/SCID +/+, −/+, and −/− mice were distinguished from H-2^u nonexpressers using PCR of gDNA with an H-2^u-specific primer pair non-cross-reactive to H-2^d expressed by the SCID founders (Fig. 2). H-2^u-positive mice were designated as homozygous for H-2^u and were used in experiments and as breeders to propagate each strain if H-2^d-specific Ab reactivity was <10% and at a low staining intensity compared with H-2^d-positive SCID mice

Table I. *Tg/SCID strain designations determined by phenotype*[a]

Phenotype[b]	Tg/SCID Strain Designation[c]			
	+/+	−/+	−/−	+/−
Surface IgM	−	−	+	+
(Fig. 1, *upper*, quad 4+)	(1 ± 1)	(2 ± 2)	(29 ± 6)	(35 ± 13)
CD4	+	−	+	+
(Fig. 1, *upper*, quad 1+)	(72 ± 17)	(1 ± 0.4)	(28 ± 9)	(30 ± 17)
Dual-positive CD4/Tg TCR	+	−	−	+
(Fig. 1, *lower*, quad 2+)	(63 ± 12)	(1 ± 1)	(1 ± 1)	(24 ± 13)

[a] Tg/SCID mice were distinguished by peripheral blood phenotype and assigned a unique designation (+ or −) for expression of the Tg TCR or the SCID defect.

[b] Surface IgM was identified by positive staining (Fig. 1, *upper row*, quad 4+ (*lower right quadrant*)) with FITC-labeled mAb specific for mouse IgM. CD4 was identified by positive staining (Fig. 1, *upper row*, quad 1+ (*upper left quadrant*) with PE-labeled mAb specific for mouse CD4. Tg TCR$^+$/CD4$^+$ donors were identified by dual-positive staining in quadrant 2 (Fig. 1, *lower row*, quad 2+ (*upper right quadrant*)).

[c] +/+, Tg TCR$^+$/SCID$^+$; −/+, Tg TCR$^-$/SCID$^+$; −/−, Tg TCR$^-$/SCID$^-$; +/−, Tg TCR$^+$/SCID$^-$. Numbers in parentheses signify the mean ± SD of the percentage of staining (isotype control background subtracted) achieved for representative groups of animals of each strain (+/+, n = 19; −/+, n = 14; −/−, n = 18; +/−, n = 60).

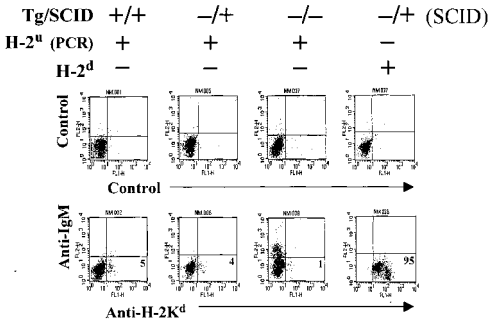

FIGURE 3. Tg/SCID mice shown to be H-2^u expressers in the PCR assay were evaluated for H-2^d expression using H-2^d-specific mAb staining. Mice with staining levels <10% of peripheral blood cells (+/+, −/+, and −/− shown) analyzed were considered to be negative for H-2^d expression. +/+, −/+, and −/− animals that were positive for H-2^d expressed at high (95%) levels (−/+ shown). Animals expressing IgM (−/−) were non-SCID.

(Fig. 3). This approach has been validated by retention of low H-2^d reactivity and an absence of H-2^u-negative offspring through subsequent generations. Mice in generations F_3–F_6 were used as study subjects in this report to evaluate the clinical course of disease.

Development of spontaneous EAE

Tg/SCID +/+ mice expressing H-2^u were followed daily for the appearance of EAE (Table II). Fifty-six percent (51 of 91) developed spontaneous ascending paralysis with an average onset at age 59 days (range, 34–106 days) and average maximal peak severity of 3.7 (range, 2–9). Disease duration cannot be inferred from these data, because different animals were removed from the study after differing periods of time. Fifty-one percent (26 of 51) of the mice that developed EAE recovered, and 38% (10 of 26) of these mice that recovered went on to develop a relapse. Eight mice that did not recover from spontaneous EAE progressed to develop a significant worsening of disease after a period of stable disease (not included in Table II as animals that relapsed after recovery). In contrast, Tg/SCID −/+ and −/− mice did not develop clinical signs of EAE. H-2^u Tg/SCID +/+ mice that received a protective injection of splenocytes from H-2^u Tg/SCID −/− donors (protected) did not develop spontaneous disease, permitting their use as breeders (Table II).

Tg/SCID +/+ mice with spontaneous EAE (51 of 91) followed several distinct patterns of disease progression (Fig. 4). Nearly all animals with spontaneous disease developed a chronic or remitting/relapsing progressive disease, and very few animals were observed to recover completely without subsequent episodes of paralysis. Using strict criteria for recovery and relapse, mice with spontaneous EAE were observed to follow four basic patterns of clinical paralysis: 1) 17 of 51 mice developed chronic mild or moderate disease without significant recovery or relapse (Fig. 4, *A* and *B*); 2) 8 of 51 mice developed chronic mild disease without significant recovery followed by significant relapse (*C* and *D*); 3) 16 of 51 mice developed mild disease followed by recovery without significant relapse (*E* and *F*); and 4) 10 of 51 mice developed moderately severe disease followed by significant recovery and significant relapse (*G–I*). Compared with Tg/SCID +/+ mice that did not develop spontaneous disease (Fig. 5*A*), Tg/SCID +/+ mice with spontaneous disease exhibited dense white matter cellular infiltrates in the spinal cord (Fig. 5, *B* and *C*).

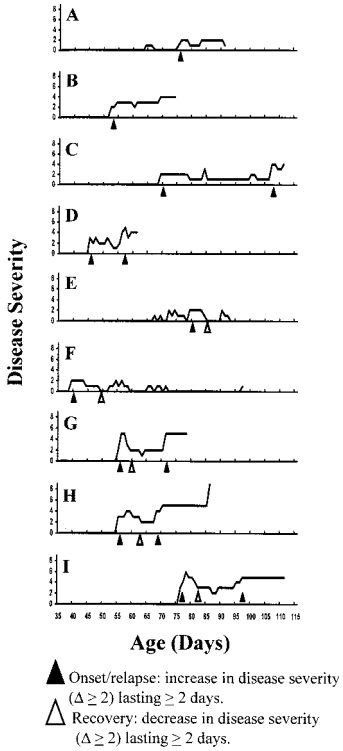

FIGURE 4. Tg/SCID +/+ mice developed spontaneous disease. Four patterns of disease from representative mice are shown: 1) chronic mild or moderate disease without significant recovery or relapse (*A* and *B*); 2) chronic mild disease without significant recovery followed by significant relapse (*C* and *D*); 3) mild disease followed by recovery without significant relapse (*E* and *F*); and 4) moderately severe disease followed by significant recovery and significant relapse (*G–I*).

Table II. *Spontaneous EAE in H-2^u Tg/SCID mice*[a]

Strain	Incidence	Onset[b]	Peak Severity[c]	Recovery[d]	Relapse[e]
Tg/SCID +/+	56% (51/91)	59 (34–106)	3.7 (2–9)	51% (26/51)	38% (10/26)
Tg/SCID −/+	0% (0/n)	NA	0	NA	NA
Tg/SCID −/−	0% (0/n)	NA	0	NA	NA
Tg/SCID +/+ protected with −/− spleen cells	0% (0/n)	NA	0	NA	NA

[a] Tg/SCID mice were followed daily for a period of over 100 days and scored (0–9 scale) for the appearance of clinical neurologic deficit. NA, Not applicable.
[b] Age of animal on first day of clinical deficit (range).
[c] Mean of the maximal score achieved (range) for each mouse in group prior to recovery; n, denominator of incidence.
[d] Animals that exhibited an improvement of ≥2 in clinical score lasting ≥2 days.
[e] Animals that exhibited a worsening of ≥2 in clinical score lasting ≥2 days following recovery. Table II does not include eight Tg/SCID +/+ animals that exhibited a significant worsening of disease (progression) following a period of stable disease without recovery.

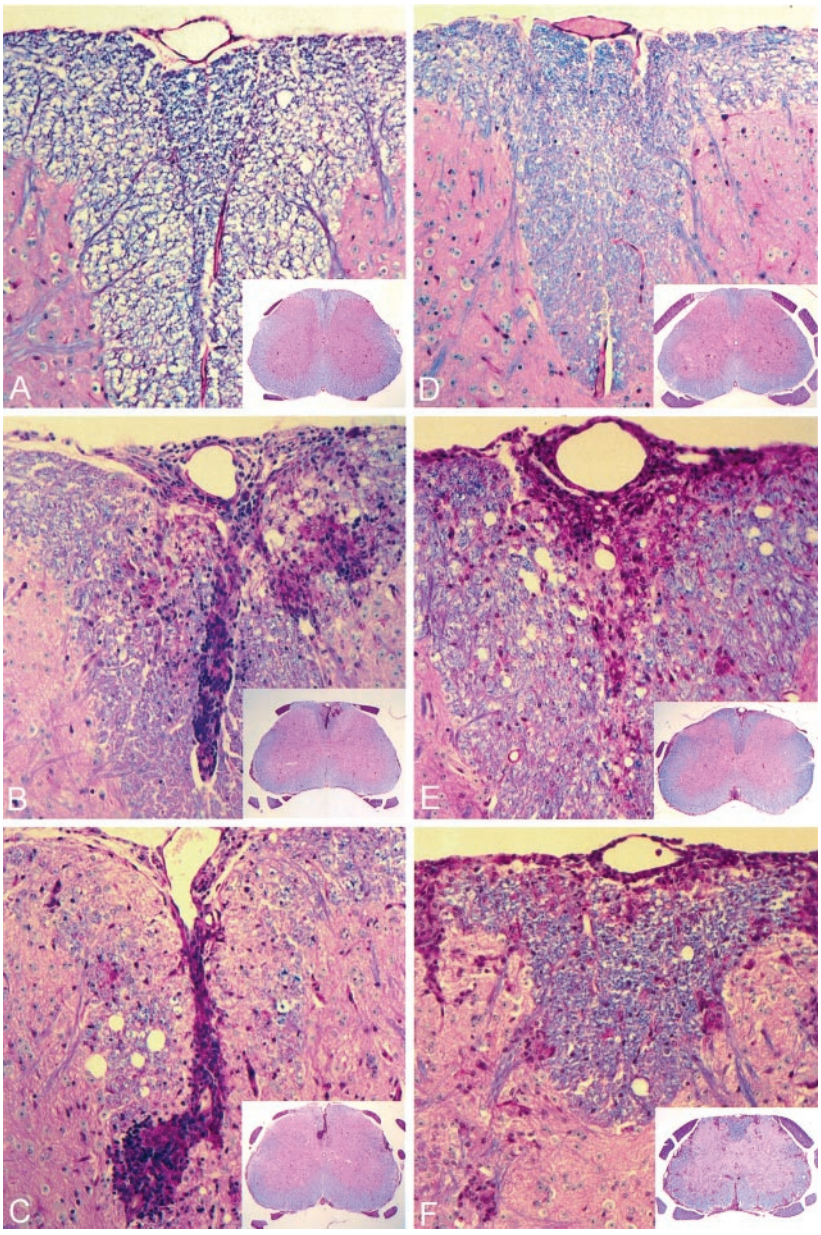

FIGURE 5. Paraffin-embedded spinal cord sections from Tg/SCID +/+ mice with spontaneous disease (*A–C*) and Tg/SCID −/− or −/+ mice with disease induced by cell transfer (*D–F*), stained with Luxol fast blue-periodic acid-Schiff-hematoxylin. *A*, No detectable clinical deficit; aged 97 days. *B*, Mild spontaneous disease followed by recovery (day 83); aged 115 days (similar to mouse shown in Fig. 4*E*). *C*, Moderately severe spontaneous disease (severity = 5) followed by recovery (severity = 2); aged 115 days. *D*, Tg/SCID −/− with very mild passive disease followed by complete recovery; specimen collected 55 days after disease induction. *E*, Tg/SCID −/+ with moderately severe acute passive disease followed by recovery and severe relapse; specimen collected 55 days after disease induction. *F*, Tg/SCID −/+ with moderately severe acute passive disease (severity = 5), no recovery; specimen collected 15 days after disease induction.

Induction of active EAE in Tg/SCID +/+ mice

Tg/SCID +/+ mice that had not developed spontaneous disease were evaluated for their ability to develop EAE following active immunization (Table III). Active immunization with $BP_{1–11}$ peptide in CFA induced EAE in 78% (7 of 9) of the Tg/SCID +/+ mice that had not already developed spontaneous disease. Disease onset occurred an average of 5.0 days (range, 1–13) after active immunization with a mean maximal disease score of 4.7 (range, 4–6). Seventy-one percent (5 of 7) of mice with peptide-induced active EAE recovered significantly, and 40% (2 of 5) of recovered mice went on to develop a significant relapse. Immunization of Tg/SCID +/+ mice with CFA alone (no peptide) induced disease

Table III. *Active EAE in H-2^u Tg/SCID +/+ mice*[a]

Immunogen	Incidence[b]	Onset[c]	Peak Severity[d]	Recovery[e]	Relapse[f]
$BP_{1–11}$/CFA	78% (7/9)	5 (1–13)	4.7 (4–6)	71% (5/7)	40% (2/5)
CFA	22% (2/9)	3.5 (2–5)	3.5 (2–5)	100% (2/2)	0% (0/2)

[a] Tg/SCID +/+ mice (average age of 92 or 94 days, respectively) without detectable spontaneous disease were immunized by s.c. injection with $BP_{1–11}$ peptide in CFA or CFA alone, followed for >45 days, and scored daily for appearance of clinical neurologic deficit.
[b] Proportion of animals with new disease during the observation period following immunization.
[c] Mean number of days following immunization on which animals first exhibited clinical deficit (range).
[d] Mean maximal score achieved (range) for each mouse in group prior to recovery.
[e] Animals that exhibited an improvement of ≥2 in clinical score lasting ≥2 days.
[f] Proportion of animals in each group that exhibited a worsening of disease following recovery.

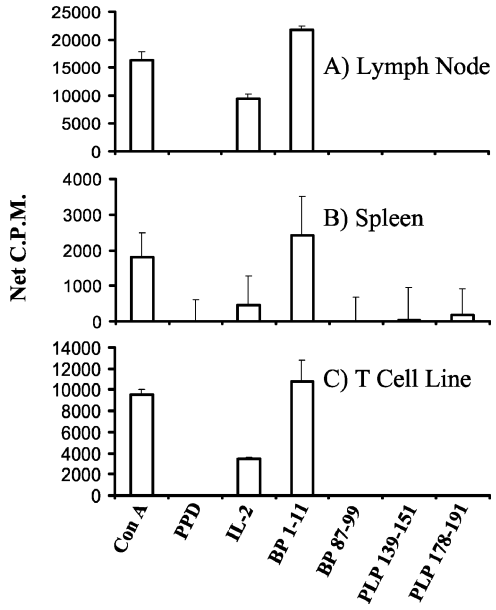

FIGURE 6. *A* and *B*, In vitro proliferative responses of LN and spleen cells isolated from immunized Tg/SCID +/+ donors 11 days after immunization with BP peptide Ac1–11 in CFA. *C*, Proliferative responses were also determined for Con A-selected T cells after expansion in IL-2 medium followed by restimulation with Con A and APC. Cells were cultured in triplicate wells for 72 h with the indicated stimulus. [^{3}H]Thymidine was added for the last 18 h of culture before harvesting and counting thymidine uptake by liquid scintillation. Results display the mean ± SD (error bars) of triplicate wells and are representative of several independent experiments.

in 22% (2 of 9) of the mice with an average onset 3.5 days (range, 2–5) after immunization and an average maximal severity of 3.5 (range, 2–5). Both of these CFA-immunized mice recovered without subsequent relapse (Table III).

Tg/SCID +/+ mice were used as immune LN and spleen donors following immunization with BP_{1-11} peptide. Freshly isolated LN and spleen cells responded specifically in vitro to stimulation with the immunizing peptide BP_{1-11} (Fig. 6, *A* and *B*, respectively) but did not respond to other myelin peptides or purified protein derivative of *M. tuberculosis*. A T cell line specific for the BP_{1-11} peptide was selected from pooled immune spleens by in vitro stimulation with the T cell mitogen Con A, followed by expansion in IL-2 medium and subsequent cycles of Con A stimulation in the presence of irradiated Tg/SCID −/− thymocyte APC and subsequent IL-2 expansion. The Con A-selected T cell line retained a focused response to the BP_{1-11} peptide (Fig. 6*C*) demonstrating a lack of additional T cell specificities in these mice.

Transfer of EAE from immunized Tg/SCID +/+ donors to −/+ recipients

Tg/SCID +/+ splenocytes stimulated in vitro with Con A were encephalitogenic in 100% of recipient mice following adoptive transfer of 20, 10, or 5×10^6 cells into immunodeficient Tg/SCID −/+ recipients (Table IV, Fig. 7). The average day of initial onset depended on the number of cells transferred: 9, 12, or 20 days post-cell transfer of 20, 10, or 5×10^6 Con A-stimulated spleen cells, respectively. The mean maximal peak severity of disease also appeared to depend on the number of T cells transferred, because recipients of 20 or 10×10^6 cells developed somewhat more severe disease (mean peak severity) than did recipients of 5×10^6 cells, and individual animals that developed mild disease (severity = 2) were observed only in the groups that received fewer cells (10 or 5×10^6). Mice that received the most cells (20×10^6) had the highest incidence of recovery and recovery followed by relapse (Table IV, Fig. 7*F*). These mice also had the most severe acute disease (as determined by time to onset, peak severity, and having the fewest animals with only mild disease). In contrast, mice that received 10×10^6 spleen cells had a low incidence of recovery (13%, 1 of 8; Table IV). Remarkably, 50% (4

Table IV. *Tg TCR +/+ donor cells transfer EAE into H-2^u SCID mice*[a]

Recipient Tg/ SCID Strain	Transferred Cells	Incidence	Onset	Peak Severity	Recovery	Relapse
−/+	20×10^6 Spleen cells[b]	100% (5/5)	9 (8–11)	6.0 ± 0.7 (5–7)	100% (5/5)	60% (3/5)[c]
−/+	10×10^6 Spleen cells[d]	100% (8/8)	12 (9–15)	6.0 ± 2.3 (2–9)	13% (1/8)	50% (4/8)[e]
−/+	5×10^6 Spleen cells[f]	100% (7/7)	20 (13–23)	4.7 + 2.1 (2–8)	71% (5/7)	29% (2/7)[g]
−/+	5×10^6 Cell line[h]	66% (2/3)	5.5 (5–6)	5.5 ± 0.7 (5–6)	0% (0/2)	0% (0/3)
−/+	2.5×10^6 Cell line[h]	100% (5/5)	8 (4–18)	6.0 + 1.2 (4–7)	0% (0/5)	20% (1/5)[i]
−/−	10×10^6 Cell line[j]	60% (3/5)	9 (9)	3.7 ± 2.1 (2–6)	66% (2/3)	0% (0/5)
−/−	5×10^6 Cell line[k]	40% (4/10)	11 (7–14)	2.8 + 0.5 (2–3)	75% (3/4)	66% (2/3)[l]

[a] The indicated number of Con A-stimulated spleen cells or Con A-selected T cell line cells from Tg/SCID +/+ spleen donors were transferred into Tg/SCID −/+ or −/− recipients.
[b] In Expt. 1, we used spleen donors designated Sply 1.
[c] Three animals relapsed after recovery. Denominator is number of recovered animals.
[d] In Expt. 2, we used spleen donors designated Sply 2.
[e] One animal relapsed after recovery. Three animals exhibited progressive worsening following a period of stable disease sufficient to satisfy criteria for relapse. Denominator is number of animals that developed spontaneous disease.
[f] In Expt. 3, we used spleen donors designated Sply 3.
[g] One animal relapsed after recovery. One animal exhibited progressive worsening following a period of stable disease sufficient to satisfy criteria for relapse. Denominator is number of animals that developed spontaneous disease.
[h] Cell line selected from Sply 3 donors.
[i] One animal exhibited progressive worsening following a period of stable disease sufficient to satisfy criteria for relapse. Denominator is number of animals that developed spontaneous disease.
[j] Cell line selected from Sply 2 donors.
[k] Cell line selected from Sply 3 donors.
[l] Two animals relapsed after recovery. Denominator is number of recovered animals.

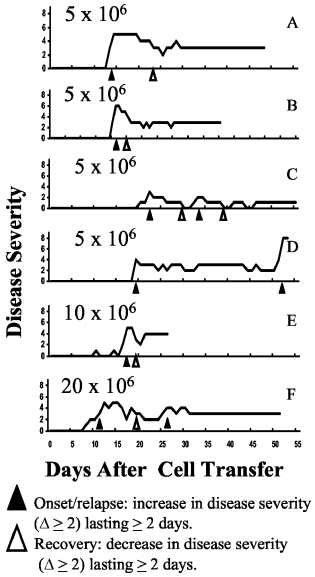

FIGURE 7. Tg/SCID −/+ mice developed EAE following passive transfer of Con A-stimulated splenocytes obtained from Tg/SCID +/+ donors. Examples of the various patterns of disease were observed following transfer of 5, 10, or 20 × 10^6 Con A-stimulated spleen cells, including: 1) mild or moderately severe disease (▲, onset) followed by recovery (△, recovery) (*A*, *B*, and *E*); 2) multiple episodes of worsening and recovery (*C*); 3) relatively stable disease followed by worsening or relapse (*D*); and 4) single cycle of acute disease followed by remission followed by relapse (*F*).

of 8) of these mice exhibited progressive worsening following a period of stable disease without recovery. This also occurred in one animal with disease induced by 5 × 10^6 cells (Fig. 7*D*).

Con A-selected Tg TCR T cell lines were also encephalitogenic in H-2^u Tg/SCID −/+ and −/− recipients (Table IV). Tg/SCID −/+ recipients were highly susceptible to disease induced by the T cell line (Sply 3) as indicated by the high incidence, early onset, and magnitude of peak severity. Cell line-mediated disease in Tg/SCID −/+ recipients was different from disease induced by Con A-stimulated spleen cells in that mice did not recover and disease progression was rare (only 1 of 5 Tg/SCID −/+ mice that received 2.5 × 10^6 line cells). Tg/SCID −/− animals were less susceptible to cell line-mediated disease as indicated by the lower incidence, longer time until onset, and lower mean peak severity even after transfer of relatively more cells compared with the −/+ recipients. In contrast to the −/+ recipients, incidence of recovery was substantial (60% (3 of 5) and 40% (4 of 10) in recipients of 10 or 5 × 10^6 line cells, respectively) and some animals (2 of 3) relapsed following recovery.

Histological examination of fixed, paraffin-embedded, Luxol fast blue-periodic acid-Schiff-hematoxylin-stained spinal cord sections from Tg/SCID mice with passive EAE (Fig. 5, *D–F*) revealed mild or no inflammation and no demyelination in a Tg/SCID −/− mouse that had recovered from mild disease (Fig. 5*D*). More severe, dense inflammation typical of EAE in the spinal cord white matter was observed in spinal cord sections from moderately and severely paralyzed mice (Fig. 5, *E* and *F*).

Discussion

Clinical paralysis followed distinct episodes of disease progression and worsening after improvement and partial recovery in a substantial proportion of Tg/SCID +/+ and −/+ mice with spontaneous or passive EAE, respectively. The absence of multiple, distinct T cells expressing multiple, distinct TCR prevented neither remissions nor disease progression in the form of distinct relapses. Conversely, myelin-specific T cells expressing only one Tg TCR were entirely sufficient for disease progression in EAE, including distinct remissions and relapses. Therefore, T cells expressing multiple distinct TCR specific for multiple distinct myelin epitopes were not required for remissions or relapses of clinical paralysis in EAE, and epitope spreading to distinct T cells specific for distinct, non-disease-initiating epitopes was not required for disease progression and relapse.

Spontaneous paralytic CNS inflammation in Tg/SCID +/+, as well as passive EAE induced with Con A-stimulated spleen cells in Tg/SCID −/+ mice, included recovery phases in a substantial proportion of the animals. The absence of recovery in Tg/SCID −/+ mice with cell line-mediated disease indicated that the cell lines and the Con A-stimulated spleen cells were not functionally identical. Thus, cells present in Tg/SCID +/+ mice with spontaneous disease and present in the transferred Con A-stimulated spleen cells from Tg/SCID +/+ donors were associated with recovery and relapse, whereas disease-inducing T cell lines were associated with neither recovery nor relapse. This raises the possibility that recovery and relapse in these mice may be related to the function of distinct cells that are present in the spleen of +/+ mice but are functionally absent or lost from the cell lines selected from spleen. Spontaneous recovery has been associated with T cell deletion mechanisms in the CNS and primary lymphoid organs as well as immune deviation in the CNS and periphery (24) in mice possessing a Tg TCR. Thus, T cell deletion mechanisms and immune deviation may be subject to regulation by cells present in the spleen of Tg/SCID +/+ mice. Moreover, distinct T cells controlling all of the distinct phases of disease—onset, spontaneous recovery, and relapse—may reside together within the spleen in Tg/SCID +/+ mice. Such functionally distinct regulatory and effector T cell populations, if present, may share a common myelin specificity with the disease-initiating cells.

The occurrence of spontaneous disease in Tg/SCID +/+ mice was similar to the occurrence of spontaneous disease in other immunodeficient mouse strains expressing an encephalitogenic Tg TCR on the B6 (H-2^u) background (25). The Tg/SCID +/+ mice reported in this study are not yet inbred, and individual, inheritable variations in susceptibility are possible (26), because the background strain used to combine the SCID phenotype with the encephalitogenic Tg TCR is an EAE-resistant strain, BALB/c (the background of C.B-17 *scid/scid* (21)). The observed variability in disease susceptibility, before achieving inbred status, represents a potential source of disease-relevant genetic variability that may be used to generate susceptible and nonsusceptible Tg/SCID +/+ strains of spontaneous EAE mice (26). Such strains might be very useful in the future for identifying gene expression profiles and/or other relevant differences associated with differences in disease susceptibility.

The relative contributions of putative specific and nonspecific immune mechanisms represent an important gap in our understanding of disease etiology in MS (1). The occurrence of spontaneous disease and susceptibility to actively induced disease in Tg/SCID +/+ mice may result from a spontaneous or induced functional absence of $CD4^+$ regulatory cells. This is suggested by reports indicating that $CD4^+$ regulatory cells function to block spontaneous disease in the presence of many encephalitogenic Tg TCR T cells (22, 27). The high incidence, moderate disease severity, and short time to onset in peptide-plus-CFA-immunized Tg/SCID +/+ mice demonstrated that mice without spontaneous disease were fully capable of developing disease after exposure to a suitable stimulus. This suggests that Ag-dependent immune sensitization in the LN may be a potentially important trigger, capable of overcoming the protective function of $CD4^+$ regulatory cells.

The requirement for CD28 in spontaneous disease in Tg TCR recombinase activating gene 1-deficient mice also implicates APC-T cell interactions, of the type that occurs during Ag-dependent T cell activation in LN, in the development of spontaneous disease (28).

Environmental triggers associated with innate immunity have been linked to spontaneous disease in certain strains possessing an encephalitogenic Tg TCR (29, 30). Such nonspecific triggers are also potentially operative in spontaneous disease in Tg/SCID +/+ mice. The differences in disease incidence and severity between Tg/SCID +/+ mice actively immunized with peptide-plus-CFA or CFA alone suggested that Ag-dependent mechanisms were more effective triggers of disease in Tg/SCID +/+ mice that had previously failed to develop spontaneous disease. Demonstrated cross-reactivity between the BP_{1-11} peptide and certain microbial peptides, including several *M. tuberculosis* epitopes (31), raised the possibility that stimulation of Tg TCR T cells following CFA immunization may have been due to the presence of a stimulatory microbial peptide. This seems unlikely because CFA alone induced disease in only a minority of the mice. However, the temporal association (3.5 days) between CFA injection and disease onset in the mice that did develop CFA-induced disease leaves open the possibility of a causal association between this form of nonspecific inflammatory stimulus and disease induction in at least some of the mice.

Whether genetic variability, environmental trigger, or some other unidentified class of stimulus is responsible for variations in spontaneous disease onset in Tg/SCID +/+ mice remains an unanswered question with high clinical relevance. In the absence of an exogenous stimulatory Ag, endogenous-Ag presentation may have provided a sufficient stimulus to initiate spontaneous disease in most Tg/SCID +/+ mice. However, in a minority of mice that did not develop spontaneous disease, this specific endogenous stimulus was either absent or insufficient, requiring an exogenous source of Ag, administered experimentally in CFA. These and other observations suggest that nonspecific environmental triggers and/or specific immune mechanisms may, under certain conditions, constitute a crucial set of overlapping processes that operate together or alone to cause the development of spontaneous disease in experimental models and MS. Such processes may also contribute to disease progression, including distinct relapses.

Endogenous-Ag presentation (or endogenous self-priming) has been proposed as a disease-dependent mechanism whereby T cells specific for spreading epitopes are triggered to participate in pathogenesis (6). In immunocompetent strains in which epitope spreading occurs, Abs and/or Ag-specific B cells may be capable of modulating Ag processing and/or presentation, respectively, in a fashion capable of directing T cell epitope reactivity toward new, spreading epitopes (32, 33). SCID mice are deficient for B cells and Ab production and such mechanisms would not be expected to operate in the Tg/SCID +/+ mice reported in this study.

One current model of disease progression in EAE proposes that disease-initiating Th1 cells enter the CNS tissue parenchyma, encounter Ag presented by CNS APC, and respond by expressing proinflammatory gene products such as cytokines. The resulting myelin destruction provides a source of new, previously unexposed Ags for endogenous-Ag presentation to new epitope-reactive cells in the CNS or in peripheral lymphoid tissues. Such newly activated T cells are proposed to induce additional CNS pathology, leading to a progression in the course of disease (13). This model predicts that distinct T cells specific for distinct multiple, spreading myelin epitopes are responsible for disease progression. However, the results presented in this study demonstrate that T cells specific for multiple myelin epitopes are not always required for disease progression and relapse. The model also predicts that disease-initiating cells are not required for disease progression and relapses. However, selective removal of disease-initiating SJL-Thy1.2$^+$ PLP-specific T cells in SJL-Thy1.1 recipient mice by in vivo treatment with anti-Thy1.2 depleting Ab was shown to block disease progression when Ab treatment occurred before or after the first or second relapse (H. Y. Tse, personal communication). Taken together, the results demonstrated that T cells specific for the disease-initiating epitope were entirely sufficient and required for disease progression and T cells specific for spreading epitopes were not required.

Discrepancies among conclusions obtained regarding the causal role for epitope spreading in the various models of progressive relapsing EAE demonstrate that an epitope-spreading model is insufficient to fully explain mechanisms controlling disease progression and relapse. A modified model of disease progression should include the following: 1) a recurring process of T cell sensitization (such as endogenous-Ag presentation) that is indirectly responsible for disease progression may likely involve T cells specific for the disease-initiating epitope (15, 16) and 2) detection of epitope spreading or lack of response by disease-initiating T cells may indicate the activity (or compartmentalization) of regulatory cells and is not necessarily a direct demonstration of crucial, disease-directing changes in the presence or activity of pathogenic T cells. Variations in the functional activity of such regulatory cells may occur independent of disease and may depend on genetic or environmental conditions inherent to a particular model or may depend on the experimental method of disease or tolerance induction (5, 11, 14, 15, 34, 35).

In summary, the underlying mechanisms responsible for relapses in EAE may, under certain conditions, involve neither epitope spreading nor lost reactivity to the disease-initiating epitope. The results provided in this study demonstrate directly that epitope spreading is not required for disease progression and relapse. Additional evidence in multiple model systems also suggests that disease-initiating cells are sufficient and necessary for disease progression and relapse. Therefore, to advance our understanding of disease progression in MS, it will be necessary to elucidate the conditions and processes responsible for disease progression in the presence and absence of epitope spreading. Then it should be possible to gain an understanding of the relative importance of the various, relevant mechanistic elements that cause disease progression.

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