***ts1*, a Temperature-Sensitive Mutant of Moloney Murine Leukemia Virus TB, Can Infect both CD4⁺ and CD8⁺ T Cells but Requires CD4⁺ T Cells in Order To Cause Paralysis and Immunodeficiency**

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When neonatal FVB/N mice were inoculated with *ts1*, a temperature-sensitive mutant of Moloney murine leukemia virus TB, they developed a progressive bilateral hindlimb paralysis and immunodeficiency leading to death 4 to 6 weeks after inoculation. T lymphocytes have been shown to be primarily responsible for this *ts1*-induced syndrome. Here we compare the role played by each subset of T lymphocytes, i.e., CD4⁺ and CD8⁺ T cells, in disease development. Mice were depleted of a specific subset for the first 10 days of their lives by using either anti-CD4 or anti-CD8 monoclonal antibodies *in vivo*. Disease development in these mice was then monitored. Depletion of CD4⁺ T cells significantly attenuated the *ts1*-induced syndrome: virus replication was decreased, disease latency was extended, and death was prevented in 60% of the mice. Similar treatment with anti-CD8 antibody had almost no effect on disease progression. However, when depletion was begun 2 weeks after neonatal *ts1* inoculation, CD4⁺ T cell depletion did not affect disease development. *ts1* infected CD4⁺ and CD8⁺ T lymphocytes equally well *in vivo*, as shown by flow cytometric analysis, but virus replication was restricted primarily to the CD4⁺ subset of T cells, as found by *in vitro* assay. Hence, CD4⁺ T lymphocytes play an important role in the development of *ts1*-induced paralysis and immunodeficiency. The mechanism of this CD4⁺ T-cell-mediated disease production by *ts1* is not clear; however, increased replication of *ts1* in the CD4⁺ T cells, especially in the early stages of the disease, seems to play a crucial role.

Newborn BALB/c and FVB/N mice, when inoculated with *ts1*, a temperature-sensitive mutant of Moloney murine leukemia virus TB, developed a syndrome characterized by progressive bilateral hindlimb paralysis and immunodeficiency (43). The advanced stages of this syndrome are associated with severe body wasting, lymphopenia, and thymic as well as splenic atrophy (26). Almost all mice die of *ts1* infection after 4 to 6 weeks (FVB/N mice) or 8 to 12 weeks (BALB/c mice) (42). However, this *ts1*-induced paralysis is age dependent; when inoculated after 10 days of age, hardly any mice developed the disease (41).

It has been shown that although normal BALB/c mice are susceptible to *ts1* infection, their athymic (nude) littermates are fully resistant to the *ts1*-induced syndrome. However, when these nude mice were given syngeneic T lymphocytes before virus inoculation, about half of the reconstituted nude mice developed the disease (26). Recently, we have shown that transient depletion of T lymphocytes in mice by using anti-Thy antibodies in the early stages of infection with *ts1* could significantly prevent disease development, whereas similar depletion of B lymphocytes had no effect on the disease profile (30). These findings clearly demonstrate that the disease syndrome caused by *ts1* depends on the presence of T lymphocytes early in life.

Almost all functional T cells in mice belong to one of two subpopulations that express the mutually exclusive surface antigens CD4 and CD8 (7, 10). Activated CD4⁺ T cells help or induce other T or B cells to proliferate, differentiate, and perform a variety of effector functions, whereas CD8⁺ T cells exhibit cytotoxic or suppressor activities (49). In this study, we investigated the role played by CD4⁺ and CD8⁺ T lymphocytes *in vivo* in disease development by *ts1*. We

selectively depleted either the CD4⁺ or CD8⁺ subset of T cells by using monoclonal antibodies (MAb) against them and followed the course of *ts1* infection in these mice. Our results show that depletion of CD4⁺ T cells for the first 10 days of life can significantly prevent the *ts1*-induced syndrome, whereas similar depletion of CD8⁺ T cells had no effect on disease development. Our results also show that although *ts1* can infect both CD4⁺ and CD8⁺ T cells *in vivo*, it replicates much better in CD4⁺ T cells.

MATERIALS AND METHODS

Virus and cells. The isolation and molecular cloning of *ts1* have been described previously (48). The virus was propagated in TB cells, a thymus-bone marrow cell line derived from CFWD mice (2), and the virus titer was determined on 15F cells as described before (44). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% fetal calf serum (FCS) and 4% bovine serum.

Antibodies. Rat MAb (immunoglobulin G2b [IgG2b]) were produced from hybridomas (obtained from the American Type Culture Collection, Rockville, Md.) GK1.5 against CD4 (L3T4) and 2.43 against CD8 (Lyt-2.2). Antibodies were harvested as ascites from primed nude BALB/c mice, partially purified by ammonium sulfate precipitations, and dialyzed against phosphate-buffered saline (PBS) as described before (39). The concentration of each antibody was measured by a radial immunodiffusion kit (ICN Immunobiological, Costa Mesa, Calif.) for quantitative determination of rat IgG2b and adjusted to a concentration of 10 mg/ml.

Infection of mice and *in vivo* depletion of T-cell subsets. The inbred FVB/N mice used in this study were obtained from the National Cancer Institute (Harlan Sprague Inc., Frederickburg, Va.). Mice were inoculated intraperitoneally

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within 48 h after birth with 0.1 ml of virus suspension containing 5×10^6 to 1×10^7 infectious units per ml. At this dose, almost all mice die within 4 to 6 weeks (42). Control animals received an identical volume of medium only.

In some experiments, a specific subset of T cells was depleted *in vivo* by intraperitoneal administration of 0.5 mg of anti-CD4 (for CD4⁺ T cells) or anti-CD8 (for CD8⁺ T cells) antibodies on days -1, +4, and +9 relative to the day of virus infection (day 0). Control mice received identical volumes of nonimmune rat serum (GIBCO Laboratories, Grand Island, N.Y.). Depletion of specific subsets of T cells was monitored by flow cytometric analysis (fluorescence-activated cell sorting [FACS]) of spleen cells for CD4, CD8, and Thy-1 surface antigens, as described below. For some mice, both infected and uninfected, anti-CD4 treatment was started at 2 weeks of age with 1 mg of antibody, followed by 0.5 mg of antibody every week to maintain depletion of CD4⁺ T cells.

Clinical study and virus assay. Neonatally inoculated mice were observed daily for the development of any clinical signs of disease for up to 120 days. Mice from each group were killed at 10-day intervals by CO₂ inhalation. Their body and thymus weights were recorded. Virus titer was measured from thymus and spinal cord homogenates on 15F cells (44). In some experiments, mice were deeply anesthetized by intravenous injection of pentobarbital (60 mg/kg) before being killed, and their blood was collected through intracardiac puncture for peripheral blood count and serum assay.

Single- and double-color immunofluorescence analysis. FACS analysis of single-cell suspensions prepared from spleens was performed on an Epics Elite flow cytometer (Coulter Electronics Inc., Hialeah, Fla.), as described before (11). *ts1* expression in the infected mice was determined by a double-color FACS analysis. Briefly, single-cell suspensions from spleens of *ts1*-infected mice at 20 days postinoculation (p.i.) were purified on a Ficoll-Hypaque gradient (Sigma Chemical Co., St. Louis, Mo.). Two million cells were incubated with goat anti-gp70 antibody (murine leukemia virus gp69/71, obtained from Microbiological Associates Inc., Bethesda, Md.). The cells were diluted 1:100 in PBS in a volume of 100 μ l on ice for 30 min and washed three times with cold PBS containing 5% FCS. Then the cells were incubated for 30 min at 4°C with fluorescein-labeled rabbit anti-goat IgG (Sigma) diluted to 10 mg/ml and either phycoerythrin-labeled anti-CD4 or anti-CD8 (both from Boehringer Mannheim Biochemicals, Indianapolis, Ind.). After an additional three washes in PBS with 2% FCS, cells were analyzed by FACS. Dead cells were excluded from analysis by setting an appropriate threshold trigger on the forward angle and 90° light scatter parameters. A minimum of 10,000 cells was counted from each sample. Samples from uninfected control mice were similarly tested for expression of any endogenous gp70. The percentages of T cells (or their subsets) were obtained similarly by direct immunofluorescence staining by FACS. The antibodies used to study the population of T cells were fluorescein-conjugated anti-Thy-1.1 (Sigma), phycoerythrin-conjugated anti-CD4 (L3T4), and anti-CD8 (Lyt2.2).

Virus replication in the separated T-cell subset. Spleen cells were isolated from *ts1*-infected mice at 20 days after infection as described above. T-cell subsets were separated as described before (12). Briefly, cells were incubated over a nylon wool column (Cellular Products, Buffalo, N.Y.) at 37°C. Nonadherent cells were eluted with L15 medium supplemented with 5% FCS. To remove CD4⁺ T cells, cells were resuspended in the GK1.5 supernatant producing anti-

CD4 antibody at a density of 5×10^6 cells per ml, incubated for 30 min over ice, and washed twice with cold medium. Similarly, to remove CD8⁺ T cells, supernatant from the 2.43 hybridoma producing anti-CD8 antibody was used. Cells were then added to washed immunomagnetic beads coated with goat anti-rat IgG (Fc-specific) antibody (Advanced Magnetics, Cambridge, Mass.) at a density of 5×10^8 cells per 10 ml of beads in a 25-cm² tissue culture flask. The flasks were gently agitated for 1 h at 4°C. Bound cells were removed with a magnet (BioMag Separator; Advanced Magnetics, Cambridge, Mass.) placed on the surface of the flask. Cells were then washed, counted, and resuspended in DMEM supplemented with 15% FCS at a density of 10^6 cells per ml and incubated at 37°C. The separation of CD4⁺ and CD8⁺ T cells was confirmed by FACS analysis. Supernatant from CD4⁺ and CD8⁺ T cells as well as from unseparated splenic mononuclear cells was collected at regular intervals and assayed for the virus titer on 15F cells (44).

Serum immunoglobulin assays. Levels of serum IgG, IgM, and IgA from infected as well as uninfected mice were determined by a radial immunodiffusion assay with a kit (The Binding Site Ltd., Birmingham, United Kingdom) according to the manufacturer's directions.

PCR. The polymerase chain reaction (PCR) was performed essentially as described by Saiki et al. (31), with slight modifications. Briefly, thymocytes were separated from 20-day-old infected or uninfected control mice after incubation overnight in plastic petri dishes to separate the nonadherent cells. Nonadherent cells were tested by FACS for T-cell receptor and Thy-1 expression. The thymocytes were washed twice in PBS and lysed at a concentration of 3×10^6 cells per ml in freshly prepared lysis buffer containing 10 mM Tris-hydrochloride (pH 8.0), 1 mM EDTA, 0.001% Triton X-100, 0.01% sodium dodecyl sulfate, and 600 μ g of proteinase K (Boehringer Mannheim Biochemicals) per ml. The lysate was digested at 57°C for 2 h, and the DNA was extracted with phenol-chloroform and precipitated with ethanol. A pair of specific primers that amplify a 1,997-bp segment of the *ts1 env* gene were constructed and used for amplification. The sequences of these primers were 5'-AT GGCGCGTTCAACGCTCTCA-3' (extending downstream) and 5'-CTATGGCTCATACTCTATAGG-3' (extending upstream). Cell clones known to be positive and negative for the *ts1 env* gene were used as positive and negative controls. DNA (1 μ g) was amplified in a 100- μ l final volume of PCR mixture. The PCR mixture contained 50 mM Tris-hydrochloride (pH 8.3); 200 mM KCl; 10 mM MgCl₂; 0.01% gelatin; 0.2 mM each dGTP, dATP, dTTP, and dCTP; 100 pmol of each primer; and 2 U of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.). The samples were overlaid with 100 μ l of mineral oil and amplified for 30 cycles in a Thermal Cycler apparatus (Perkin Elmer Cetus) that provided 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C per cycle. At the end of 30 cycles, the apparatus was programmed to add one extra cycle and polymerization at 72°C for 10 min to ensure completion of the amplified products. The PCR products were detected by agarose (1.5%) gel electrophoresis and staining with ethidium bromide.

RESULTS

Effect of *in vivo* CD4⁺ or CD8⁺ T-cell depletion on *ts1* disease pathogenesis. A single injection of 0.5 mg of anti-CD4 or anti-CD8 MAb in newborn mice followed by two similar injections over the following 9 days almost completely depleted the specific subset of T cells in the periphery.

TABLE 1. FACS analysis of spleen cells from uninfected and *ts1*-infected mice^a

Mice	Treatment	Age (days)	% Positive cells		
			Thy-1.1 ⁺	CD4 ⁺	CD8 ⁺
Uninfected	Normal rat serum	10-12	78.4	48.2	12.6
		35-40	84.2	50.6	13.1
	Anti-CD4 MAb	10-12	28.2	1.6	10.8
		35-40	68.6	42.5	12.1
	Anti-CD8 MAb	10-12	68.8	46.1	1.0
		35-40	74.6	44.2	8.9
<i>ts1</i> infected	Normal rat serum	10-12	76.5	50.0	10.6
		35-40	61.8	33.3	14.9
	Anti-CD4 MAb	10-12	30.6	2.2	10.5
		35-40	60.6	40.2	13.5
	Anti-CD8 MAb	10-12	74.8	50.6	1.4
		35-40	58.8	32.4	12.9

^a Mice were the same as in Fig. 1. Results are the averages for three to four mice for each group.

However, when the mice were 35 to 40 days old, a significant proportion of the depleted T cells returned to the periphery (Table 1). Treatment of 2-week-old mice with 1 mg of anti-CD4 MAb reduced the concentration of CD4⁺ T cells in the periphery to less than 2% within 48 h. This depletion was maintained by weekly injection of 0.5 mg of antibody until the end of the experiment (data not shown).

Depletion of CD4⁺ T cells for the first 10 days of life had a strong protective effect against *ts1*-induced death, whereas similar depletion of the CD8⁺ T cells had almost no effect on disease progression. CD4⁺ T-cell-depleted, *ts1*-infected mice remained healthy and survived longer than CD8⁺ T-cell-depleted, *ts1*-infected mice or positive control mice (treated with normal rat serum and infected with *ts1*). As shown in Fig. 1, 60% of CD4⁺ T-cell-depleted, *ts1*-infected mice survived for more than 120 days, whereas all mice from the CD8⁺ T-cell-depleted, *ts1*-infected or the positive control group died by 40 days p.i. When treatment with antibodies or nonimmune serum alone was evaluated in groups of uninfected mice, no significant difference from untreated mice was observed (data not shown).

Body and thymus weights from mice of different groups were compared. Both body and thymus weights were significantly higher in CD4⁺ T-cell-depleted, *ts1*-infected mice and uninfected control mice than in the CD8⁺ T-cell-depleted, *ts1*-infected and positive control mice, especially after 20 days (Fig. 2). However, the weights were somewhat higher in the uninfected control mice than in the CD4⁺ T-cell-depleted, *ts1*-infected mice. Mice neonatally infected with *ts1* and depleted of their CD4⁺ T cells after 2 weeks of age did not show any noticeable difference in disease progression or thymus and body weights compared with normal mice infected with *ts1* (data not shown).

Virus titers in the thymus and spinal cord were measured in all groups of mice at regular intervals. As shown in Table 2, 15- to 100-fold fewer virus were observed in the CD4⁺ T-cell-depleted, *ts1*-infected mice than in the CD8⁺ T-cell-depleted, *ts1*-infected or positive control mice. The difference was more pronounced in the early stages of the infection. However, no significant difference was observed

FIG. 1. Survival of uninfected and *ts1*-infected FVB/N mice under different treatments. Ten mice from each group were infected with *ts1* within 48 h after birth and treated with 0.5 mg of either anti-CD4 or anti-CD8 MAb or normal serum on days -1, 4, and 9. Groups: uninfected control (○); anti-CD4 treated, *ts1* infected (▲); anti-CD8 treated, *ts1* infected (●); normal serum treated, *ts1* infected (△).

between the titers of CD8⁺ T-cell-depleted, *ts1*-infected and positive control mice at any time point.

Significant differences in the peripheral blood profile were observed between CD4⁺ T-cell-depleted, *ts1*-infected and CD8⁺ T-cell-depleted, *ts1*-infected mice in the advanced stages of the disease. Acute lymphopenia was observed in the positive control and CD8⁺ T-cell-depleted, *ts1*-infected mice. Only a slight reduction in the lymphocyte count was noticed in CD4⁺ T-cell-depleted, *ts1*-infected mice (Table 3). The lymphopenia observed was mainly due to the reduction in the CD4⁺ T-cell population, as determined by FACS analysis. The ratio between CD4⁺ and CD8⁺ T cells changed from 3.9 in the age-matched uninfected control group (50.8% CD4⁺/12.8% CD8⁺) to 2.4 in the terminal stages of *ts1*-infected mice (34.2% CD4⁺/13.9% CD8⁺).

Productive infection of thymocytes and peripheral CD4⁺ T cells by *ts1*. To determine *ts1* integration in the thymus, we performed a PCR amplification of the *ts1 env* gene in a highly purified population of thymocytes (more than 99% positive for T-cell receptor and Thy-1; data not shown) from infected and uninfected mice. As shown in Fig. 3, a specific amplified product of the *ts1 env* gene of about 2 kb was observed in *ts1*-infected mice (lane 2), whereas no specific product was revealed in uninfected age-matched control mice (lane 1). An infectious-unit assay showed that virus titers similar to those

TABLE 2. Titer of *ts1* in organs of FVB/N mice receiving normal serum (PBS) or anti-CD4 or anti-CD8 antibody^a

Time p.i. (days)	Virus titer (log ₁₀ I.U./g of tissue homogenate)					
	Thymus			Spinal cord		
	PBS	Anti-CD4	Anti-CD8	PBS	Anti-CD4	Anti-CD8
10	7.8×10^5	9.1×10^3	9.2×10^5	1.2×10^5	1.0×10^3	9.8×10^4
20	9.0×10^7	2.1×10^6	8.8×10^7	3.6×10^7	5.8×10^5	4.6×10^7
30	8.6×10^7	5.4×10^6	6.7×10^7	6.2×10^7	9.6×10^5	7.8×10^7

^a Mice were treated as described in the legend to Fig. 1. Virus titers were determined on 15F cells as described before (44) and are expressed as averages for three to four mice per group. I.U., infectious units.

obtained from unseparated splenic cells (described below) were produced by primary-cultured thymocytes obtained from *ts1*-infected mice (data not shown).

Next, we examined *ts1* replication in the periphery in total splenic mononuclear cells as well as in each T-cell subset. T cells were separated into CD4⁺ CD8⁻ and CD4⁻ CD8⁺ subsets by negative selection from *ts1*-infected spleens.

FIG. 2. Effect of treatment with anti-CD4 or anti-CD8 antibodies on body (A) and thymus (B) weights of *ts1*-infected mice. Newborn mice were given three injections of anti-CD4 (□), anti-CD8 (●), or normal serum (○) as in Fig. 1 and infected with *ts1* within 48 h after birth. Δ, uninfected age-matched control. Each point represents an average for three to four mice per group.

FACS histograms demonstrated that our separation technique with magnetic beads resulted in a depletion efficiency of greater than 96%, leaving a population of negatively selected cells consisting primarily of either CD4⁺ or CD8⁺ T cells (data not shown). Cells (10^6 /ml) from each group were incubated, and at regular intervals, supernatant was assayed for production of infectious virus. CD4⁺ T cells produced the highest titer of virus, which gradually rose until the second day, after which it dropped slightly. Unseparated cells produced a titer that was close but not quite equal to that produced by CD4⁺ T cells. CD8⁺ T cells produced a basal level virus titer that was 100- to 500-fold lower than that produced by CD4⁺ T cells or unseparated cells (Fig. 4). These results suggest that CD4⁺ T cells are the primary replication sites for *ts1* in the spleen.

Expression of *ts1* in both CD4⁺ and CD8⁺ T cells in vivo. To determine the expression of *ts1* in vivo, we used double-color FACS analysis of the splenic cells. Figure 5 shows the percentages of CD4⁺ and CD8⁺ cells that expressed the virus. Of the total splenic cells, 4.2% expressed both *ts1* (gp70) and CD4 (Fig. 5B), whereas 1.4% coexpressed *ts1* and CD8 (Fig. 5D). In repeated experiments, coexpression of *ts1* and CD4 ranged from 4 to 6%, whereas that of *ts1* and CD8 ranged from 1 to 1.5%. Therefore, in both the CD4⁺ and CD8⁺ subsets of T cells, about 1 in 10 cells expressed *ts1* at 20 days p.i., since the normal ratio of CD4⁺ and CD8⁺ cells was about 3.9 in FVB/N mice (see above). Figures 5A and C show normal percentages of CD4⁺ and CD8⁺ cells, respectively, in uninfected mice and that no significant endogenous gp70 was expressed in the uninfected control mice.

Effect of depletion of CD4⁺ or CD8⁺ cells on hyperglobulinemia produced by *ts1* infection. We have previously reported abnormalities of B cells, e.g., hyperglobulinemia, in *ts1*-infected mice (41). To determine whether depletion of T cells early in life had any effect on the hyperglobulinemia

TABLE 3. Absolute counts of leukocytes, lymphocytes, and neutrophils in peripheral blood from FVB/N mice^a

Animal group	Avg absolute count (10^3 /mm ³)		
	Leukocytes	Lymphocytes	Neutrophils
Uninfected control	7.65	6.96	0.61
<i>ts1</i> infected (positive control)	2.60	1.41	0.94
<i>ts1</i> infected, anti-CD4 treated	6.46	5.49	0.77
<i>ts1</i> infected, anti-CD8 treated	3.20	1.61	1.10

^a Mice were treated as described in the legend to Fig. 1. All mice were tested at an age of 35 to 40 days. Values represent the averages for three to four mice per group. Total leukocyte counts were performed on a Coulter S770 blood counter. Differential counts were performed on blood smears by standard methods. Absolute counts equal the total count multiplied by the decimal percentage of the differential counts.

FIG. 3. Presence of viral DNA in the thymocyte population of *ts1*-infected mice. One microgram of DNA from thymocytes of uninfected control mice (lane 1), thymocytes from *ts1*-infected mice (lane 2), an NIH 3T3 cell clone 1 (48) expressing the *env* gene of *ts1* (lane 3), *ts1*-infected TB cells (lane 4), and uninfected TB cells (lane 5) was amplified by PCR as described in Materials and Methods, with specific primers that amplify a 1,997-bp segment of the *ts1 env* gene. Lane 6 is the *Hind*III-digested lambda DNA size marker ladder.

found in *ts1*-infected mice, we tested the levels of IgM, IgG, and IgA in all groups of mice. As shown in Table 4, a significant increase (more than threefold) in the level of IgG and a moderate increase in the level of IgM were observed in *ts1*-infected (positive control) mice as well as in CD4⁺ T-cell-depleted, *ts1*-infected and CD8⁺ T-cell-depleted, *ts1*-infected mice, without much variation among these groups. However, no significant change was observed in the IgA levels in any group of mice.

DISCUSSION

T lymphocytes play a vital role in host immunity (4, 12, 19, 20). However, in many viral infections, T cells or their subsets are found to be directly or indirectly responsible for causing the disease rather than preventing it (23, 36). This study compares the role of helper (CD4⁺) and cytotoxic (CD8⁺) T cells in the pathogenesis of *ts1*-induced paralysis

FIG. 4. Infectious units (I.U.) of *ts1* in total mononuclear cells (cross-hatched bars), CD4⁺ T cells (open bars), and CD8⁺ T cells (solid bars) obtained from spleens of *ts1*-infected mice. FVB/N mice were infected with *ts1* within 48 h after birth and killed 20 days p.i., and mononuclear cells, CD4⁺ T cells, and CD8⁺ T cells were isolated from spleens, as described in Materials and Methods. The number of I.U. in each cell population in the supernatant was then determined at the indicated times. Results are expressed as log₁₀ I.U./10⁶ viable cells plated.

FIG. 5. Expression of *ts1* on CD4⁺ and CD8⁺ subsets of T lymphocytes. The number of cells expressing both *ts1* and T-cell surface antigen (either CD4 or CD8) is shown as double color in the upper right-hand quadrant of each panel. Ficoll-purified mononuclear cells from spleens of *ts1*-infected (20 days p.i.) (panels B and D) or age-matched uninfected control (panels A and C) mice were incubated first with goat anti-gp70 and then with fluorescein isothiocyanate-labeled anti-goat IgG and phycoerythrin-labeled anti-CD4 (panels A and B) or anti-CD8 (panels C and D) MAb, as described in Materials and Methods. The upper left-hand quadrant of each panel depicts the percentages of CD4⁺ cells (A and B) or CD8⁺ cells (C and D) not expressing any gp70 on the surface.

and immunodeficiency in FVB/N mice. We provide evidence that the presence of CD4⁺, not CD8⁺, T cells in the first few days of life is primarily responsible for the development of the syndrome induced by *ts1*. However, both CD4⁺ and CD8⁺ T cells can be infected by *ts1*, although the virus replicates primarily in the CD4⁺ subset of T cells.

FVB/N mice are highly susceptible to *ts1*-induced paralysis and immunodeficiency (42). Previously, we showed that the thymus and T cells are primarily responsible for this *ts1*-induced disease syndrome (26, 30). In this study, we examined the role of CD4⁺ and CD8⁺ T cells in *ts1*-induced pathogenesis by directly depleting each subset of T cells for the first 10 days of life by administering MAb. Transient depletion of CD4⁺ T cells for the first 10 days of life had a

TABLE 4. Immunoglobulin levels in *ts1*-infected FVB/N mice^a

Mice ^b (no. in group)	Mean concn ± SD (μg/ml)		
	IgM	IgG	IgA
Normal uninfected (4)	397 ± 18	585 ± 101	196 ± 39
<i>ts1</i> infected (positive control) (4)	487 ± 31	1,787 ± 141	221 ± 84
<i>ts1</i> infected, anti-CD4 treated (4)	523 ± 16	1,638 ± 86	187 ± 13
<i>ts1</i> infected, anti-CD8 treated (4)	571 ± 42	2,010 ± 48	212 ± 32

^a All mice tested were 35 to 40 days old. Concentrations of each isotype were determined with a radial immunodiffusion kit for mouse antibodies as described in Materials and Methods.

^b Same as in Fig. 1.

significant protective effect against *ts1*. Mice depleted of CD4⁺ T cells survived much longer (Fig. 1), were healthier (Fig. 2), had a much longer latency period, and did not develop severe lymphopenia (Table 3) compared with positive control mice. Similar depletion of CD8⁺ T cells had little or no effect on the disease process.

To examine whether the presence of CD4⁺ T cells was important not only to initiate but also to sustain disease development by *ts1*, mice were infected neonatally and the disease was allowed to develop for 2 weeks before treatment with anti-CD4 antibody was started. Although CD4⁺ T cells were efficiently depleted in these mice, no significant change was observed in the disease development by this treatment (data not shown). This observation showed that CD4⁺ T cells are essential for *ts1* only in the early stages of disease development.

Apart from T cells, CD4 is also expressed on some macrophages (13, 33). In fact, CD4-mediated infection of macrophages is assumed to be an important factor in the damage of the central nervous system (CNS) in human immunodeficiency virus infection (37). We previously showed that macrophages could be productively infected by *ts1* in primary culture (43). However, CD4 expression in macrophages is species specific; it has been shown that mouse macrophages do not express CD4 (9). Therefore, prevention or attenuation of the *ts1*-induced neurodegeneration by treatment with anti-CD4 antibodies cannot be attributed to any direct role of macrophages.

Although the thymus and T cells have been shown to play an important primary role in *ts1*-induced disease (26, 30), a productive infection of thymocytes or T cells in *ts1* infection had not been shown before this study. The presence of the *env* gene in the PCR-amplified product of purified thymocytes from *ts1*-infected mice (Fig. 3) together with the release of infectious virus particles from primary thymocyte culture clearly showed that productive infection of thymocytes is an integral part of *ts1* infection. A similar assay for released virus from a primary splenic cell culture of infected mice showed that primarily CD4⁺ T cells produced infectious virus and that the majority of the virus found in the supernatant of unseparated splenic cells was probably produced by CD4⁺ T cells (Fig. 4). The virus assay from cultured cells is a sensitive technique but depends on cells producing infectious virus. The type and number of cells expressing viral protein *in vivo* was determined by double-labeled FACS analysis (Fig. 5). Since the percentages of *ts1* (gp70)-expressing cells were similar within each CD4⁺ and CD8⁺ T-cell population, as evident from the FACS study, it is possible that although *ts1* infects both CD4⁺ and CD8⁺ T cells equally well, *in vivo* it replicates primarily in the CD4⁺ T cells. A significantly higher number of CD4⁺ T cells (about 1 in 10) were infected, as determined by FACS analysis, compared with the number determined by the infectious-unit assay (Fig. 4), which showed that only 1 in 300 to 2,000 cells was producing infectious virus. Although the lower number of infected cells detected by the infectious-unit assay may reflect the low plating efficiency of T cells, this is probably not the sole reason for this difference. These results suggest that at any given time, a substantial number of infected CD4⁺ T cells were not producing infectious virus. Ahmed et al. (1) reported the same phenomenon in lymphocytic choriomeningitis virus (LCMV) infection of lymphocytes. Productive infection of a small fraction of lymphocytes was enough to cause human immunodeficiency virus-induced AIDS (reviewed in reference 29). Similar observations were

made with LCMV (24), murine AIDS virus (47), and human cytomegalovirus (28) infections.

ts1 causes acute lymphopenia and thymic atrophy in the advanced stages of the disease (26, 30). Recently, we have shown that the thymic atrophy seen in *ts1* infection is a direct effect of virus replication and that T-cell killing is a property of *ts1* that is not shared by its parental wild-type virus (45). In this study, we have further shown that the lymphopenia observed in *ts1* infection is due primarily to the depletion of CD4⁺ T cells in the terminal stages of the disease (from 50.8% to 34.2%). Although *ts1* replicates well in CD4⁺ T cells in the early stages of the disease, its killing of these CD4⁺ T cells in the terminal stages of the disease is an important feature of *ts1* infection that is shared by human immunodeficiency virus (18). A similar phenomenon was also observed in persistent infections with some LCMVs (24). However, the mechanism(s) of virus-induced CD4⁺ T-cell depletion is still not well understood. A single amino acid change has recently been shown to be responsible for allowing a variant of LCMV to induce immunosuppression and persistent infection (32). A point mutation that causes a Val-25 to Ile substitution in *ts1* has been shown to be responsible for causing paralysis and immunodeficiency in FVB/N mice (42). The same mutation is also responsible for causing nonprocessing of gpPr80^{env} at the restrictive temperature (35). The defect in processing of gpPr80^{env} is probably due to the inability of a precursor polyprotein to be transported out of the endoplasmic reticulum, resulting in its accumulation in the cell (15, 34, 35). This defective processing may be important for the cytopathic effects observed in *ts1* infection in CD4⁺ T cells or neural cells, which eventually lead to disease development. Inefficient processing of *env* precursor polyprotein has been shown to be responsible for cell death in some cases of feline leukemia virus (25) and human immunodeficiency virus (16) infections. We are currently investigating the role of gpPr80^{env} in cell killing in primary cultures of T lymphocytes and glial cells.

Involvement of CD4⁺ T cells has been implicated in the pathogenesis of many diseases, including autoimmune diseases such as experimental allergic encephalomyelitis (38), murine lupus erythematosus (40), collagen arthritis (27), and myasthenia gravis (8), as well as many virally induced diseases (1, 3, 36, 47). Oldstone and coworkers (1, 5, 24) have shown the probable role of CD4⁺ T cells in enabling the cytomegalovirus to avoid the host's immunologic surveillance machinery in persistent LCMV infections. Murine retrovirus-induced immunodeficiency syndrome (MAIDS) induced by LP-BM5 viruses is thought to be caused by an abnormal response of infected CD4⁺ T cells, which in turn affects B cells; however, virus replication is not essential in the development of MAIDS (reviewed in reference 14). On the contrary, measles virus, although it infects both T and B lymphocytes, causes immunosuppression by acting directly on B cells (22). Infection with but not necessarily replication of virus was required for immunosuppression caused by murine cytomegalovirus infection (6).

An indirect and possibly autoimmune role is played by CD4⁺ T cells in encephalomyocarditis virus-induced encephalomyelitis (36). Our previous studies have shown the absence of any signs of inflammation in the CNS in *ts1*-induced paralysis (43). By using different mutants, we have also shown that the injury to the nervous system corresponds to the mutant's increased ability to replicate in the CNS (34). Here we report a significant decrease in the virus titer (15- to 100-fold) in both the thymus and CNS by depleting CD4⁺ T cells for the first 10 days of life (Table 2).

The virus replicates primarily in CD4⁺ T cells in the early stages of the disease (Fig. 4). It is feasible that damage to the thymus or CNS is caused directly by the increased level of virus replication. Therefore, depletion of CD4⁺ T cells in the early stages of *ts1* infection reduced the virus load and consequent damage to the CNS and thymus. In normal mice neonatally infected with *ts1*, the virus reaches a titer high enough to overwhelm the rather immature immune system and invade the CNS within 2 weeks p.i.; therefore, depletion of CD4⁺ T cells at or after that time point did not affect disease progression. This fact also supports the hypothesis that *ts1*-induced neurodegeneration is probably not caused by an autoimmune reaction. We cannot completely rule out the possibility of CD4⁺ T-cell-mediated release of a neurotoxic factor(s), e.g., tumor necrosis factor alpha, which may play an important role and cause injury to the CNS in the early stages of *ts1* infection. In vivo treatment with anti-CD4 antibodies depleted peripheral CD4⁺ T cells but did not deplete the CD4⁺ cells in the thymus (39). Therefore, the fact that treatment with anti-CD4 antibody also decreased thymic virus titers indicated that (i) CD4⁺ T cells are important not only for virus replication but also for virus dissemination and/or (ii) the binding of anti-CD4 antibody in the thymus may somehow prevent the infection of thymocytes, most of which, including the earliest T-lineage precursor cells, express CD4 antigen (46). This may also explain why treatment with anti-CD4 antibodies could not completely stop *ts1* infection, as virus replication in the thymus plays a major role in *ts1* pathogenesis and virus production may have continued in the thymus after treatment with anti-CD4 antibodies. Recently, Korostoff et al. (17) have shown that thymic tolerance in mice neonatally infected with Gross murine leukemia virus plays a crucial role in the induction of immunologic nonresponsiveness. A similar mechanism cannot be ruled out in *ts1* pathogenesis, since development of tolerance could be prevented by treatment with anti-CD4 antibody in newborn mice (21).

The increased level of immunoglobulin secretion in all groups of infected mice showed that it was probably caused by direct stimulation of B cells and not mediated via helper T cells, as seen in MAIDS (47). Increased levels of IgG and IgM were observed in all mice infected with *ts1*, irrespective of whether they were treated with anti-CD4 or anti-CD8 antibodies (Table 4). However, the highest level of immunoglobulins was observed in the CD4⁺ T-cell-depleted, *ts1*-infected group of mice killed after 120 days, which never developed any clinical sign of disease (data not shown). This shows that the hyperglobulinemia present following *ts1* infection may have nothing to do with the development of CNS disorders. The role of this hyperglobulinemia is not clear at present.

In conclusion, the data presented in this article show that CD4⁺ T cells play a crucial role in *ts1*-induced paralysis and immunodeficiency, whereas CD8⁺ T cells have little or no role in pathogenesis. At present, the mechanism(s) by which CD4⁺ T cells cause this immunoneurologic disorder is not clear; however, they appear to provide a favorable site for *ts1* replication during the early stages of infection. Why CD4⁺ T cells are more prone to increased virus replication than CD8⁺ T cells, especially in the early stages of infection, is unknown. Whether other indirect factors, e.g., an increased level of cytokine secretion or secondary infection of other cell types, may play an important role in *ts1* infection is not known. The implications of our results for the possible relationship between immune and neurological disorders caused by retroviruses are as yet not clear. It may well prove

profitable to further study the role of CD4⁺ T lymphocytes in inflicting injury to the nervous system in *ts1* infection.

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