Brief Definitive Report

MOUSE THYMIC VIRUS (MTLV)

A Mammalian Herpesvirus Cytolytic for $CD4^+$ ($L3T4^+$) T Lymphocytes

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In 1961, Rowe and Capps (1) described a murine herpesvirus, now known as mouse thymic virus (MTLV; ICTV designation murid herpesvirus 3), which caused severe thymic necrosis and acute immunosuppression in newborn mice (1–4). Although the thymus and the affected peripheral lymphoid tissues regenerate within 1–2 mo after infection, infected animals continue to shed virus for life (1, 3). MTLV is a good candidate for a T lymphocytolytic virus. Virus and viral antigen are present in infected thymuses (3), and T lymphocytes may be required for infection (5). However, little is known about specific T lymphocyte target cells in vivo for lymphotropic herpesviruses.

Mouse thymocytes can be phenotyped using mAbs to the principal T lymphocyte surface markers CD4 (L3T4) and CD8 (Lyt-2) (6). These surface markers define four major thymocyte subpopulations ($CD4^+8^+$, $CD4^+8^-$, $CD4^-8^+$, and $CD4^-8^-$). In the mouse, most thymocytes are immature cortical double-positive ($CD4^+8^+$) cells, representing 80–90% of the total, while mature functional thymocytes are $CD4^+8^-$ (approximately equivalent to human T4 helper/inducer T lymphocytes) or $CD4^-8^+$ (6). We here report that the $CD4^+$ cell is the major lytic target in the thymus for MTLV infection.

Materials and Methods

Mice. Mice, obtained from The Rockefeller University Laboratory Animal Research Center barrier breeding colony (BALB/c) or from Taconic Farms, Germantown, NY (Taconic Swiss), were free of murine pathogens including MTLV (4). Mice had food and water ad libitum, and were treated in accordance with National Institutes of Health guidelines.

Virus. For virus pools (3), litters of newborn (≤24 h old) BALB/c or Taconic Swiss mice were inoculated intraperitoneally (30-gauge needle) with 0.05 ml MTLV per neonatal mouse. Thymuses were harvested 7 d later and 10% (wt/vol) homogenates were prepared in culture medium (DME or Iscove's; Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (HyClone Laboratories, Logan UT). The virus pool was stored at −70°C after the addition of DMSO (Sigma Chemical Co., St. Louis, MO) to a final concentration of 10%. Virus titer was generally 3.5–4.2 $\log_{10}$ ID_{50} per ml as calculated by the Spearman-Kärber method (7). For virus titration, serial 10-fold dilutions of virus were inoculated into litters of newborn mice (0.05 ml per mouse). The pups were examined at 9 d for macroscopic thymic necrosis. Control homogenates of uninfected thymuses did not induce thymic necrosis.

Thymocyte Preparation and Analysis. Newborn mice were either left uninfected (control) or

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infected with ~50 ID_{50} of MTLV per mouse. Thymocytes were harvested by standard methods (8). Briefly, thymuses from each group of suckling mice were collected, placed into sterile 60-mm tissue culture dishes (Corning, Corning, NY) containing 1 ml Iscove's medium with 10% FCS, and disaggegated with forceps. The suspensions were transferred to sterile 15-cc conical polypropylene centrifuge tubes (Corning), diluted to 5 ml with medium, allowed to settle for 5 min, and the supernatant fluid was then transferred to new tubes. After viable counts in isotonic 0.04% trypan blue (Gibco Laboratories), 10^6 cells from each sample were transferred to 1.5 ml polypropylene microcentrifuge tubes (Fisher Scientific Co., Springfield, NJ), gently pelleted in a microcentrifuge (Fisher Model 59A, 5 min at setting 2) and washed once with HBSS (Gibco Laboratories). For staining, fluorescent labeled rat mAbs to mouse CD4 (anti-L3T4 phycoerythrin conjugate, Becton Dickinson & Co., Mountain View, CA) and CD8 (anti-Lyt-2 fluorescein conjugate, Becton Dickinson & Co.) were mixed together at a 1:40 dilution in HBSS. Each cell pellet (10^6 cells) was gently resuspended in 100 μl of the antibody preparation and incubated for 45 min at 4°C. After incubation, the cells were diluted to 1 ml with Iscove's medium containing 1% FCS, gently pelleted, washed once with additional medium, and resuspended in 0.5 ml of the same medium. Control thymocytes after processing were >90% viable by trypan blue count. Cells were analyzed by flow cytometry within 2 h, except for experiments in Table II, where stained cells were fixed at 4°C in freshly prepared 2% paraformaldehyde (9) and kept at 4°C in the dark for analysis within 1 wk. Comparisons of fresh vs. paraformaldehyde-fixed samples indicated only minimal changes in the cells. Two-color flow cytometric analysis, using a minimum of 10,000 cells for each determination, was performed with a B-D FACS 440 cell sorter (Becton Dickinson & Co., Mountain View, CA) equipped with Spectra-Physics laser. Except for Fig. 1, dead cells were removed by gating, with gates set using control thymocytes (>90% viable).

Results

Thymic necrosis resulted, as expected (3, 4), in virtually all inoculated suckling mice. Macroscopic necrosis peaked at ~8–11 d. Infected thymuses were generally <10% of normal size and weight. Some infected thymuses were only moderately necrotic; these were generally ~25% of normal size.

Infection caused a marked decrease in both subpopulations of CD4 bearing thymocytes (both $CD4^+8^+$ and $CD4^+8^-$) (Fig. 1, *B–C*), as compared with controls (Fig. 1 *A*). At the peak of necrosis, $CD4^+8^+$ cells were reduced considerably below control levels, by a degree that appeared to depend on severity of necrosis (Fig. 1, *B* and *C*). $CD4^+8^-$ cells were even more strikingly reduced, to 1% or less of total thymocytes in all cases. The major surviving lymphocytes were $CD4^-8^+$ and $CD4^-8^-$ cells. These effects were not strain specific. Similar results were obtained with Taconic Swiss mice.

Calculations of yields of each cell population per thymus revealed that while $CD4^-8^+$ and $CD4^-8^-$ populations appeared to increase relative to the other populations, their absolute numbers were not increased (Table I). In contrast, $CD4^+8^-$ cells were reduced to 2% or less of control.

To determine whether there were any differences in the two $CD4^+$ populations ($CD4^+8^-$ and $CD4^+8^+$), numbers of $CD4^+$ cells in the thymus were monitored during the first week of infection, before gross thymic necrosis was visible. As shown in Table II, both populations remained at control levels for the first 3 d after infection. $CD4^+8^-$ cells began to decline on the fourth day after infection, while $CD4^+8^+$ cells appeared to remain at normal levels through day 5, then declined. $CD4^+8^+$ cells on day 5 were present at 98% of control levels; total cell yields and thymic appearance were normal. Day 6 thymuses were macroscopically normal, but analysis indicated that both $CD4^+$ cell populations were largely nonviable by then.

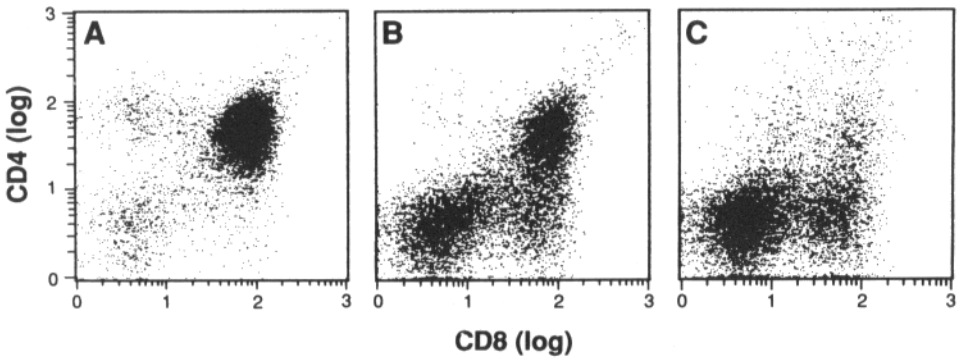

FIGURE 1. Two-color immunofluorescent flow cytometric analysis of thymocyte subpopulations in MTLV-infected mice. Log fluorescence intensity of staining with anti-CD8-fluorescein (*x* axis) and anti-CD4-phycoerythrin (*y* axis) of thymocytes from control BALB/c suckling mice (*A*) or from MTLV infected mice (8 d after infection) showing moderate thymic necrosis (*B*) or severe thymic necrosis (*C*). Each cell is represented by one dot. Thymócyte subpopulations (with data gated for live lymphocytes) calculated as percent of total thymocytes remaining in each population:

	Percentage of total thymocytes with phenotype:			
Mice	$CD4^+8^-$	$CD4^+8^+$	$CD4^-8^+$	$CD4^-8^-$
(*A*) Control	3.8	89.0	2.9	2.6
(*B*) Infected (moderately necrotic)	0.7	58.1	23.1	15.2
(*C*) Infected (severely necrotic)	1.0	19.8	38.2	38.3

Discussion

The data presented here indicate that MTLV infection is preferentially cytolytic for $CD4^+$ cells. $CD4^+8^-$ cells in the thymus were virtually eliminated irrespective of the apparent severity of the infection. $CD4^+8^-$ cells, unlike $CD4^+8^+$ thymocytes, are immunocompetent cells (6). The selective loss of $CD4^+8^-$ cells (as compared with $CD4^-8^+$ cells) correlates well with the immunosuppression that was reported to occur during acute infection with thymic virus (2), which involved loss of helper cell activity, but not of suppressor activity.

TABLE I

Absolute Numbers of Each Thymocyte Subpopulation in MTLV-infected Mice

	Number of cells per thymus				
Mice	Total yield	$CD4^+8^-$	$CD4^+8^+$	$CD4^-8^+$	$CD4^-8^-$
Control	3.1×10^8	1.2×10^7	2.8×10^8	9.4×10^6	8.4×10^6
Infected (moderate necrosis)	2.4×10^7	1.7×10^5	1.4×10^7	5.7×10^6	3.7×10^6
Infected (severe necrosis)	5.1×10^6	5.3×10^4	1.0×10^6	2.0×10^6	2.0×10^6

Thymuses from control and infected mice (moderately or severely necrotic) were harvested 8 d after infection and thymocytes were prepared and analyzed as for Fig. 1. Percentages of cells in each population, as determined by two-color flow cytometry (gated data), were multiplied by the viable count (in 0.04% trypan blue) for each sample to obtain absolute yields of each population per thymus

TABLE II

Changes in Thymocyte Subpopulations during Course of Infection

Days after infection	$CD4^+8^-$		$CD4^+8^+$	
	Total	Control	Total	Control
	%		%	
2	9.6	97.8	76.8	96.1
3	8.2	99.2	79.4	102.0
4	6.7	81.3	83.7	102.0
5	3.0	72.5	80.1	97.9
6	1.6	29.2	14.3	20.1

Thymocytes from age-matched control and infected mice (Taconic Swiss strain) were harvested and phenotyped on the days indicated.

In contrast to the $CD4^+$ populations, $CD4^-8^+$ and $CD4^-8^-$ cells appeared largely to be spared. Gating should have excluded dead cells from being miscounted as $CD4^-8^-$. Mosier et al. (10) found that >90% of thymocytes from both MTLV infected and normal thymuses also stained identically for Ly-1, Ly-2 (CD8), and Ly-3.

The loss of CD4 bearing cells also appears specific to this virus, and not a generalized effect of damage to the thymus. Several other treatments reported to cause thymic atrophy, including malnutrition (11) and mouse cytomegalovirus infection concurrent with induced graft-vs.-host reaction (12), showed virtually normal CD4/CD8 subpopulation distributions even when total thymocyte numbers were greatly reduced.

Because MTLV is present in infected thymuses (3), cytolytic infection appears the most likely explanation for thymocyte loss after MTLV, but alternative mechanisms are possible. MTLV could inappropriately trigger normal thymic mechanisms for clonal elimination of self-reactive T lymphocytes (13), or primary infection of nonlymphocyte cells by the virus could result in loss of thymocytes as an indirect effect. Glucocorticoid induction during infection is also a possible mechanism (14), but it appears unlikely to be responsible for the loss of the more mature $CD4^+8^-$ cells, which are generally considered cortisone resistant (8).

It is intriguing to speculate from the results that the CD4 molecule may be the virus receptor, as it is for HIV 1 (15, 16), but the evidence is insufficient to support this hypothesis. Our preliminary results from cell sorting experiments suggest instead that at least some $CD4^-$ cells may become infected with the virus but survive. These surviving cells could remain infected and serve as a reservoir of chronic infection with prolonged virus shedding. The cell type in which the virus persists is unknown, but other evidence implicates a T lymphocyte population (5).

There are likely to be other T lymphocytolytic viruses in nature with a similar predilection for CD4 bearing cells. One possible parallel is the recently described human herpesvirus 6 (HHV-6), also known as HBLV (17). Originally isolated from B lymphocytes, HHV-6 was recently demonstrated able to cytolytically infect a human T lymphoblastoid line in vitro (18), although cytolytic infection of T lymphocytes by HHV-6 has yet to be demonstrated definitively in vivo. I have found growth of MTLV in several T lymphoblastoid lines tested, including three $CD4^+$ human lines (Morse, S. S., manuscript submitted for publication). HHV-6 has other similarities to MTLV, including asymptomatic infection and chronic virus shedding in saliva

(19). MTLV and HHV-6 might be the first identified members of a family of lymphotropic, T cytolytic herpesviruses.

Summary

Mouse thymic virus (MTLV; ICTV designation murid herpesvirus 3) infects developing T lymphocytes of neonatal mice, causing thymic necrosis and acute immunosuppression. Infected animals shed virus indefinitely. In the present report, two-color flow cytometric analysis of T lymphocyte subpopulations defined by the markers CD4 (L3T4) and CD8 (Lyt-2) was used to determine whether MTLV was lytic for a specific thymocyte population. At peak necrosis (8–11 d after infection), numbers of $CD4^+8^+$ cells in the thymus were reduced by 80% or more as compared with controls, and $CD4^+8^-$ cells were reduced by >98%. The major survivors were $CD4^-8^+$ and $CD4^-8^-$ lymphocytes. These data indicate that the CD4 bearing lymphocyte is a primary target for cytolysis during MTLV infection. Possible parallels between MTLV and a newly described lymphotropic human herpesvirus, human herpesvirus 6 (HHV-6/HBLV), are also suggested.

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