Viral Infection of the Thymus

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We have examined infection of the thymus during congenitally acquired chronic lymphocytic choriomeningitis virus (LCMV) infection of mice, a classic model of antigen-specific T-cell tolerance. Our results show that (i) infection starts at the fetal stage and is maintained throughout adulthood, and (ii) this chronic infection of the thymus can be eliminated by transfer of virus-specific cytotoxic T lymphocytes (CTL) that infiltrate the thymus and clear all viral products from both medullary and cortical regions. Elimination of virus from the thymus results in abrogation of tolerance. During the fetal stage, the predominant cell type infected is the earliest precursor of T cells with a surface phenotype of Thy1+CD4+CD8+J11d+. In the adult thymus, infection is confined primarily to the corticomedullary thymocytes present in the medullary region. The infected cells are CD4+ and J11d+. The presence of J11d, a marker usually associated with immature thymocytes, on infected single positive CD4+ "mature" thymocytes is intriguing and suggests that infection by this noncytolytic virus may affect development of T cells. There is minimal infection of the CD8+ medullary thymocytes or of the double positive (CD4+ CD8+) cells present in the cortex. Infection within the cortex is confined to the stromal cells. Interestingly, there is infection of the double negative (CD4− CD8−) thymocytes in the adult thymus, showing that even during adulthood the newly developing T cells are susceptible to infection by LCMV. Virus can be eliminated from the thymuses of these carrier mice by adoptive transfer of LCMV-specific CTL. The transferred CTL infiltrate the thymus and clear all viral products from the medullary region first and then from the thymic cortex. This result clearly shows the need to reevaluate the widely held notion that mature T cells are unable to reenter the thymus. In fact, in our experiments the donor T cells made up to 20 to 30% of the total cells in the thymus at 5 to 7 days after the transfer. The number of donor T cells declined as virus was eliminated from the thymus, and at 1 month posttransfer, the donor T cells were hardly detectable. The results of this study examining the dynamics of viral infection and clearance from the thymus, the primary site of T-cell development, have implications for understanding tolerance induction in chronic viral infections.

The thymus is the primary site of positive and negative selection events that shape the T-cell repertoire (9, 11, 22, 27, 29). Despite the importance of this organ in the development of T cells and the well-established role of cellular immunity in controlling viral infections, there have been relatively few studies examining involvement of the thymus during a viral infection (5, 30). In this study, we have addressed this issue using the murine model of infection with lymphocytic choriomeningitis virus (LCMV).

Mice infected with LCMV at birth or in utero become lifelong carriers with high levels of virus in most of their organs, including the thymus (6, 18). These carrier mice do not exhibit a generalized immune suppression and have normal T- and B-cell responses to most antigens but have a selective defect in generating T-cell responses against LCMV (6, 18). We have examined infection of the thymus in this model of antigen-specific T-cell tolerance. Specifically, we have asked the following questions. (i) At what stage does the thymus first get infected in congenital LCMV carriers? (ii) What cell types are infected and where these infected cells are located in the thymus? In addition, we have examined the kinetics and pattern of viral clearance from the thymus following immune therapy and monitored the infiltration of virus-specific cytotoxic T lymphocytes (CTL) into the thymus.

MATERIALS AND METHODS

Mice. BALB/cByJ (H-2k), C57BL/6 (H-2b; Thy1.2), and B6.PL Thy1.1/Cy (H-2b; Thy1.1) mice were purchased from Jackson Laboratory, Bar Harbor, Maine. The congenitally infected LCMV carrier colonies were bred and established at the University of California at Los Angeles. Carrier mice 6 to 8 months old were used for experiments. Pregnant carrier mice at 14 to 18 days of gestation were used for the fetal thymus studies. LCMV immune mice were made by injecting 6- to 12-week-old BALB/cByJ or B6.PL Thy1.1/Cy mice intraperitoneally with 105 PFU of LCMV. At 30 to 90 days postinfection, these mice were sacrificed and their spleens and lymph node cells were used as LCMV-specific immune cells.

Virus. The Armstrong CA 1371 strain of LCMV (Arm-7) and a variant derived from this virus (Arm-13) were used in this study (4). Arm-7 was used in all experiments involving BALB/c mice, whereas Arm-13 was used to establish the C57BL/6 carrier colony.

Virus titrations. Infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (4).

Antiserum. The following monoclonal antibodies were used for identifying the various T-cell subsets. (i) HO-13-4 (anti-Thy1.2) and J11d were obtained from the American Type Culture Collection (Rockville, Md.) and grown in our laboratory. (ii) AD4-15 (anti-CD8) was purchased in the form of
ascitic fluids from Cedarlane Laboratories (Hornby, Ontario, Canada) and used at concentrations specified by the supplier. (iii) The hybridoma producing RL172.4 (anti-CD4) was provided by Mike Bevan, Scripps Clinic and Research Foundation (La Jolla, Calif.) and 1:5 dilution of its supernatant was used. (iv) Anti-Thy.1.1 and anti-Thy.1.2 conjugated to fluorescein isothiocyanate were obtained from ICN Immunobiologicales (Lisle, Ill.) and used at concentrations specified by the supplier.

Identification of thymocytes infected with LCMV. Single-cell suspensions of fetal, newborn, and adult thymus were obtained for depletion of specific T-cell subsets by treatment with monoclonal antibody plus complement using procedures previously described (3, 16). Virus was detected by infectious center (IC) assay and immunofluorescence. In each experiment, fetal or newborn thymus tissues were collected simultaneously with adult thymus for comparison and identification of cell type infected.

Immunofluorescence and immunoperoxidase staining. Frozen thymus tissue was cut to 6-µm-thick sections then fixed in steps in 95% ethanol–absolute ether (1:1 volume) and 95% ethanol. After two washes in phosphate-buffered saline, the sections were then stained with polyclonal guinea pig anti-LCMV serum (1:100 dilution). The polyclonal anti-LCMV serum recognizes all of the major structural proteins of LCMV and shows no reactivity with uninfected cells or tissues from uninfected mice. The guinea pig anti-LCMV serum was followed by fluorescein-conjugated rabbit anti-guinea pig immunoglobulin G (1:100 dilution) (Cooper Biomedical, Inc., West Chester, Pa.) when immunofluorescence staining was desired. For immunoperoxidase staining, peroxidase-conjugated affinity-purified goat anti-guinea pig immunoglobulin G (heavy plus light chains) (Accurate, Westbury, N.Y.) (1:100 dilution) was added as the second antibody. This was followed by incubation with the substrate 3,3′-diaminobenzidine (Sigma, St. Louis, Mo.) in a solution containing 0.1 M Tris buffer (pH 7.6), 1.0% (vol/vol) normal goat serum, and 0.1% (vol/vol) of 3% hydrogen peroxide.

Immune therapy of LCMV carrier mice. A total of 2 × 107 to 3 × 107 spleen and lymph node (LN) cells obtained from B6.PL Thy1+ (H-2b; Thy1.1) mice injected 30 to 90 days previously with LCMV were transferred intraperitoneally into 6- to 12-week-old C57BL/6 (H-2b; Thy1.2) carrier mice. In experiments with BALB/c carrier mice, the donor lymphocytes were obtained from BALB/c immune mice. To monitor for viral clearance, thymuses from treated carrier mice were harvested at several time points posttransfer and assayed for the presence of infectious virus by plaque assay and for viral antigen by immunofluorescence and immunoperoxidase staining.

LCMV-specific CTL assay. Virus-specific CTL in the thymus were determined by a 6-h 51Cr release assay as previously described (4).

RESULTS

Identification of cell types infected in the thymuses of carrier mice. Infection of the thymus during chronic LCMV infection has been previously documented (2, 6, 18). However, the stage at which virus spreads to the thymus and the cell types that harbor LCMV have not been characterized. To address this issue, we have examined the thymuses of congenitally infected BALB/c mice during various stages of life (fetus, newborn, and adult).

(i) Fetal thymus. Fetuses from pregnant LCMV carrier mice between 14 and 18 days of gestation were used to check infection of the thymus. Single-cell suspensions of the thymus made, and cells producing virus were scored by an IC assay. In three separate experiments, we found that between 0.5 to 1.0% of the fetal thymocytes were productively infected. To determine the surface phenotype of infected cells, the thymocytes were treated with various antisera plus complement before they were plated on Vero cells for the IC assay. The effect of antiserum treatment on the number of ICs produced by day 14 fetal thymus cells is shown in Fig. 1. Treatment with complement plus anti-Thy1.1 or anti-J11d resulted in ≥90% decrease in the number of LCMV ICs, indicating that the majority of infected cells were positive for the Thy antigen and also expressed J11d, which is a marker for immature T cells. Treatment of these day 14 fetal thymocytes with anti-CD4 and anti-CD8 plus complement had no effect, showing that the infected cells were of the double negative (DN) (CD4+ CD8−) phenotype. The results shown in Fig. 1 clearly demonstrate that infection of the thymus in congenital LCMV carriers starts at the fetal stage and the predominant cell type infected is the earliest precursor of T cells with a surface phenotype of Thy1+ CD4+ CD8− J11d+. It should be noted that at 14 days of gestation the fetal thymus consists mostly (about 90%) of these DN thymocytes (9, 11, 22, 27, 29).

(ii) Newborn thymus. The thymuses of 1-day-old (<24-h) congenital carrier mice were checked for presence of virus. We found that about 1 to 2% of the thymocytes were productively infected. The phenotype of these cells was quite heterogeneous; there was infection of DN cells and cells expressing CD4 and CD8. In these experiments, it was not determined whether the CD4+ and CD8+ infected cells were double positive (DP) or single positive (SP) thymocytes. The presence of LCMV-infected CD4+ and/or CD8+ T cells in 1-day-old congenital carrier mice suggests that the type of cells infected is influenced by the changes occurring in the thymus as development progresses from the fetal to the newborn stage. It is well established that the percentage of DN thymocytes decreases with concomitant increases in the fractions of DP and SP cells as the development proceeds from the fetal stage to the newborn stage (9, 11, 22, 27, 29).

(iii) Adult thymus. Infection of the thymus during adult-
hood was monitored by checking several (>30) congenital carrier mice ranging in age from 1 to 4 months old. Similar results were seen in all experiments and between 1 to 3% of the thymocytes were releasing infectious virus. A representative set of results obtained by the IC assay are shown in Fig. 2 and Table 1. It is clear from these data that in the adult thymus, infection is confined primarily to the SP CD4+ thymocytes. Although these SP CD4+ thymocytes make up only about 5 to 10% of the total cells in the thymus, a substantial fraction (about 20%) of these CD4+ cells were infected with LCMV (Table 1). In contrast, there was minimal (<0.1%) infection of the DP (CD4+ CD8+) cells which constitute the bulk (about 80%) of the thymus. There was also minimal infection of the SP CD8+ thymocytes. It is worth noting that in the adult thymus, where the DN (CD4- CD8-) T cells make up only a small (about 5%) percentage of the total cells, a fraction of these early T cells harbored virus. This result is significant, since it shows that even during adulthood the newly developing T cells are susceptible to infection by LCMV. It is also worth noting that the virally infected SP cells expressed J11d, a marker usually associated with immature and nonfunctional thymocytes (9, 11, 22, 27, 29). The presence of J11d on infected SP "mature" thymocytes suggests that infection by this noncytolytic virus may be affecting the development and function of these T cells.

We next determined whether infection in the thymus was located within the medullary and/or cortical regions. To answer this question we used two approaches: (i) hydrocortisone treatment to deplete thymus cortex and then check the number of LCMV-infected cells, and (ii) immunofluorescence staining of thymus sections with virus-specific antisera. Since cortical (immature) thymocytes are sensitive to hydrocortisone and medullary (mature) thymocytes are resistant to this treatment, we examined the effect of hydrocortisone treatment on the number of LCMV-infected cells in the thymus. Adult carrier mice were injected intraperitoneally with 5 mg of hydrocortisone, and their thymuses were analyzed 2 days later. Hydrocortisone treatment depleted the cortical regions and resulted in a 10-fold reduction in the number of thymocytes but had only a minimal effect on the number of LCMV-infected thymocytes (average of 5.9 × 10^6 infected cells in the thymuses of control mice [n = 4] versus 4.4 × 10^5 infected cells in the thymuses of hydrocortisone-treated mice [n = 4]). Similar results were seen in a second experiment. Thus, these results show that infection is confined primarily within the hydrocortisone-resistant mature thymocytes. Immunofluorescence staining also confirmed heavy infection in the medullary region (Fig. 3A and B). However, it is worth noting that the cortical region is also positive for virus and the infection appears to be in stromal cells.

**Clearance of viral antigen from the thymus.** Our previous studies have shown that infectious virus can be eliminated from the thymuses of carrier mice by adoptive transfer of T cells from LCMV immune mice (2, 14, 15). To examine the kinetics of viral clearance from the medullary and cortical regions, we checked the levels of infectious virus and viral antigen in the thymus at different time points (day 8, 12, 16, 22, and 30) after adoptive transfer. In untreated adult carrier mice, there is heavy infection of the medullary region with some involvement of the cortical region. See examples shown in Fig. 3A and B. These samples contained >10^5 PFU of LCMV per organ. At 8 days after adoptive transfer, an obvious change in the distribution of viral antigen was noted. There was some reduction in virus infection of the medullary region, but there was still substantial virus in the cortical areas (Fig. 3C and D). At this time, there was no detectable infectious virus (<50 PFU per organ) despite the presence of viral protein. At day 12, viral antigen was still readily detectable in the thymus. By days 16 and 22 after immune therapy, only trace viral antigen could be found and by day 30, all the viral antigen was gone with complete clearance (data not shown). In other words, persistent viral infection can be cleared from both the medullary and the cortical regions of the thymus in about 3 to 4 weeks after immune therapy.

To determine whether the transferred LCMV-specific CTL infiltrate the thymus, we injected 3 × 10^7 spleen cells from Thy1.1 immune mice into Thy1.2 carrier recipients and quantitated the percentage of donor T cells in the carrier

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**TABLE 1. Quantitation of thymocyte subsets infected with LCMV in adult congenital carriers**

<table>
<thead>
<tr>
<th>Thymocyte subset</th>
<th>Total no. (%) of cells/thymus</th>
<th>No. of infected cells/thymus</th>
<th>% Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>DN CD4- CD8-</td>
<td>3.5 × 10^9 (5.0)</td>
<td>6.1 × 10^6</td>
<td>1.74</td>
</tr>
<tr>
<td>DP CD4+ CD8+</td>
<td>5.7 × 10^8 (82.6)</td>
<td>4.7 × 10^5</td>
<td>0.08</td>
</tr>
<tr>
<td>SP CD4+</td>
<td>6.7 × 10^6 (9.7)</td>
<td>1.2 × 10^5</td>
<td>17.91</td>
</tr>
<tr>
<td>SP CD8+</td>
<td>1.8 × 10^6 (2.6)</td>
<td>1.4 × 10^4</td>
<td>0.77</td>
</tr>
<tr>
<td>Total</td>
<td>6.9 × 10^7</td>
<td>1.3 × 10^6</td>
<td>1.88</td>
</tr>
</tbody>
</table>

* Thymocytes obtained from three 2-month-old congenital carriers were pooled and separated into the various subsets by a combination of positive and negative selection techniques as described in Materials and Methods. The purity of each subset population was then confirmed by fluorescence-activated cell sorting analysis. The number of cells within each thymocyte subset producing virus was quantitated by an IC assay.
thymus at 0, 5, 7, 14, and 28 days after immune therapy. We
found that the donor T cells can be detected as early as 5
days after immune therapy (Table 2). At this time, between
5 to 28% of the total cells in the thymus were of donor origin,
and interestingly, the majority of the infiltrating cells were
CD8+ T cells. In the three mice examined, the number of
infiltrating CD8+ T cells were two- to threefold greater than
the number of CD4+ donor T cells. This is particularly
noteworthy, since the original population of spleen cells
transferred contained more CD4+ T cells than CD8+ T cells
/about 1.5:1 ratio). The number of infiltrating cells in the
carrier thymus remained quite high at day 7 (22 to 31%) and
again showed a preponderance of CD8+ T cells. The number
of donor T cells were lower at day 14 posttransfer and were
barely detectable at day 28. Functional assays showed that
the infiltrating donor CD8+ T cells contained LCMV-specific
CTL (Table 3). Taken together, these results show that
virus-specific CTL infiltrate the thymus within 5 days after
transfer, most likely proliferate as a result of antigen-specific
stimulation, eliminate virus from both medullary and cortical
regions in about 3 to 4 weeks, and then exit from the thymus
as the virus is eliminated.

**DISCUSSION**

In this study we have examined infection of the thymus
during congenitally acquired chronic LCMV infection of

**TABLE 2. Quantitation of transferred T cells present in the
carrier thymus**

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>0</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Thy1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>% CD4+</td>
<td>2</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>% CD8+</td>
<td>8</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>% CD4+</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

A total of 3.0 × 10^7 spleen cells from Thy1.1 LCMV immune mice were
transferred into Thy1.2 LCMV carrier mice. At the indicated times, mice were
sacrificed and the number of donor Thy1.1 T cells present in the carrier
thymus was determined by staining with fluorescein isothiocyanate-conju-
gated anti-Thy1.1 monoclonal antibody and fluorescence-activated cell sorting
analysis. The day 5 and day 7 samples were also double stained with anti-CD4
and anti-Thy1.1 and with anti-CD8 and anti-Thy1.1 to determine the subset of
infiltrating donor T cells.
mice, a model of antigen-specific T-cell tolerance. We show that (i) infection starts at the fetal stage and is maintained throughout adulthood, and (ii) this chronic infection of the thymus can be eliminated by transfer of virus-specific CTL that infiltrate the thymus and clear all viral materials from both medullary and cortical regions.

Infected during the fetal stage is predominantly of the DN thymocytes (CD4− CD8−), the earliest precursor of T cells. It was not established whether these cells first become infected within the thymus or whether the stem cells coming from the bone marrow already harbor virus. However, it is clear that T cells become infected with LCMV during their earliest stage of development. Although infection of T cells in LCMV carrier mice has been documented, this is the first report identifying the developmental stage at which T cells become infected (3, 8, 26, 28). It is particularly noteworthy that even during adulthood, when these DN cells make up only a small percentage (~5%) of the total thymocytes, some of these early T cells are infected. This shows that there is continuous infection of newly developing T cells throughout the life of the carrier animal. These results suggest that the constant level of infected T cells seen in the peripheral organs of carrier mice is maintained by continuous infection of newly developing T cells.

Infection in the adult thymus was predominantly of the cortisone-resistant CD4+ T cells found in the medullary region. Almost 15 to 20% of these cells were productively infected with LCMV. In contrast to the heavy infection of the CD4+ medullary thymocytes, there was only minimal (~<1%) infection of the CD8+ T cells in the thymus. This finding mirrors the results seen with infection of peripheral T-cell subsets in carrier mice, about 5 to 10% of CD4+ T cells being infected with minimal involvement of CD8+ T cells (3, 28). The results of this study show that the pattern of infection seen among the peripheral T-cell subsets is already established within the thymus. It is somewhat perplexing why there are fewer infected CD8+ T cells, especially if infection is first initiated at the DN stage. It is possible that following infection of DN thymocytes viral gene expression is selectively repressed in cells that develop into the CD8+ lineage, perhaps by production of an antiviral product made only by the CD8+ subset. Alternatively, our findings raise the intriguing possibility that the DN progenitors for the two T-cell subsets may come from different pools and that the DN thymocytes that give rise to CD4+ T cells are more sensitive to LCMV infection than the DN cells that develop into CD8+ T cells. Another possibility is that the viral infection is down-modulating expression of the CD8 molecule and/or affecting the development of this T-cell subset (i.e., infected DN thymocytes are impaired in their ability to give rise to CD8+ T cells). Admittedly, these ideas are highly speculative; however, it is worth noting that the virally infected medullary (“mature”) CD4+ T cells expressed J11d (Fig. 2), a marker usually associated with immature and nonfunctional thymocytes (9, 11, 22, 27, 29). The presence of the J11d marker on infected SP “mature” thymocytes suggests that infection by this noncytolytic virus may be affecting the development and function of these T cells.

This study shows that virus-specific CTL infiltrate the thymus and eliminate virus from medullary and cortical regions. This finding is of interest, since reentry of mature T cells into the thymus is considered to be a rare event and a widely held notion among immunologists is that T-cell traffic from the thymus is strictly unidirectional (i.e., only out and not in) (9, 11, 22, 27, 29). However, a few studies have shown that transferred T-cell lines or activated T cells can localize in the thymic medulla (1, 12, 20, 23, 24). A recent study has shown that antivirally active T cells can enter the thymus during an acute viral infection (10). Thus, the results of our study, along with these other reports, seriously question the dogma about the inability of T cells to reenter the thymus. It is clear from our studies that virus-specific CTL can infiltrate the thymus and eliminate virus. It is particularly interesting that virus is also eliminated from the cortical region, an area of the thymus where migration of transferred T cells has not been previously documented.

The thymus is the primary site of T-cell development and repertoire selection (9, 11, 22, 27, 29). Hence, an important consequence of infection of this organ during the fetal or neonatal period is the induction of T-cell tolerance to the microbe. Our results show that the thymus first becomes infected during the fetal stage and thus right from ontogeny, when tolerance to self is developing. LCMV is like a “self” antigen for these mice. This finding is consistent with the observation that in vivo virus-specific T-cell tolerance seen in carrier mice and with studies showing clonal deletion of virus-specific CTL (4, 25). We have shown that following elimination of virus from the thymus by immune therapy, the congenitally infected carrier mice that were previously tolerant to the virus acquire LCMV-specific CTL responses as a result of export of new T cells (13, 15). These results show that infection of the thymus during the fetal stage is not by itself sufficient to induce a permanent state of tolerance and that continuous infection of the thymus during adulthood is necessary to maintain T-cell tolerance to a virus. Our finding that continuous infection of the thymus is crucial in maintaining the tolerant state may explain why complete immunological tolerance is not always seen in perinatally acquired chronic infections.

It will be of interest to examine other models of viral persistence to determine whether infection of the thymus correlates with the level of tolerance to the infectious agent. Korostoff et al. (17) have shown that neonatal exposure of mice to Gross murine leukemia virus results in infection of thymocytes as well as thymic stromal cells and such mice exhibit T-cell tolerance to the virus. There are some inter-
testing natural veterinary infections characterized by antigen-specific T-cell unresponsiveness, such as bovine viral diarrhea virus, for which the issue of thymus involvement needs to be addressed (7). Among viruses persisting in humans, it will be particularly useful to examine infection of the thymus in hepatitis B virus (HBV) infections. HBV infections that occur in adults are usually acute and resolved within a few months. However, the vast majority of infants born to infected mothers (HBV envelope antigen positive) are likely to become carriers. This striking difference in the outcome of HBV infection is believed to reflect the immunological status of the host at the time of initial infection, and there is evidence to suggest that perinatal exposure to HBV results in T-cell tolerance to certain viral proteins (21). The problems of T-cell tolerance and thymus infection also need to be addressed with human immunodeficiency virus, especially given the fact that perinatal infection is increasingly becoming a significant mode of transmission of this virus (19).

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