Virus-Lymphocyte Interaction: T Cells of the Helper Subset Are Infected with Lymphocytic Choriomeningitis Virus during Persistent Infection In Vivo

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The lifelong persistence of lymphocytic choriomeningitis virus (LCMV) in neonatally or congenitally infected mice is accompanied by a suppression of virus-specific T-cell responses. In this study, we identified the subset of T cells infected with LCMV during persistent infection in vivo. Using specific monoclonal antibodies to separate the different lymphocyte cell populations and employing both an infectious center assay and immunofluorescence to detect the virus, we found that infection is confined primarily to T cells of the helper subset (L3T4⁺ Lyt2⁻), with minimal involvement of cytotoxic T cells (Lyt2⁺ L3T4⁻) and mature B cells. About 0.54 to 1.1% of L3T4⁺ T cells were producing the virus, as determined by the infectious center assay. In contrast, 9.1 to 12.2% of these L3T4⁺ T cells contained viral antigen, as shown by immunofluorescence studies. This finding suggested that, at any given time, a substantial number of infected T cells were not producing infectious virus. This infection of T helper cells may be involved in the suppression of LCMV-specific T-cell responses observed in persistently infected mice.

The ability of viruses to infect lymphocytes and macrophages has important biological implications. This interaction with the cells of the immune system may play a role in the spread and dissemination of infection within the host, in viral latency and persistence, in the transmission of virus to uninfected individuals, and may, in part, be responsible for the immunosuppression that is seen in many viral infections (17).

Infection of mice with lymphocytic choriomeningitis virus (LCMV) is an excellent experimental model for the study of virus-lymphocyte interaction during persistent infection in vivo (3, 13, 18). Mice infected with LCMV at birth or in utero become lifelong carriers, with high levels of infectious virus in most of their major organs. Persistence of LCMV in these carrier mice is accompanied by T-cell unresponsiveness to the virus. Such persistently infected mice show limited or no detectable cytotoxic-T-lymphocyte (CTL) or delayed-type-hypersensitivity responses against LCMV, and they also show either a paucity or a lack of T cells that will proliferate when stimulated with the virus (1, 3, 13, 18; R. Ahmed and L. Butler, unpublished data). The underlying mechanism(s) of this suppression of LCMV-specific T-cell responses is not fully understood. Earlier studies by Doyle and Oldstone (7) and Popescu et al. (19) have shown that a small proportion of the T cells of carrier mice are infected with LCMV. However, the subset of T cells infected with the virus was not known.

T cells can be divided into two broad classes on the basis of their expression of the cell surface molecules L3T4 and Lyt2. Most T helper cells, which provide help for antibody production and generation of CTLs, are L3T4⁺ Lyt2⁻, whereas most CTLs are Lyt2⁺ L3T4⁻ (4, 6). These two T-cell subsets also differ in their pattern of H-2 restriction; L3T4⁺ Lyt2⁻ cells recognize antigen in association with H-2 class II (Ia) molecules, while Lyt2⁺ L3T4⁻ cells are restricted primarily by H-2 class I (H-2K, H-2D) molecules (6,

24). In this study, we have identified the subset of T cells that harbor LCMV during persistent infection in vivo; our results show that the virus is present in L3T4⁺ Lyt2⁻ T cells, i.e., in cells with the surface phenotype characteristic of T helper cells.

MATERIALS AND METHODS

Mice. BALB/c ByJ mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. The congenitally infected LCMV carrier colony was established and bred at the University of California, Los Angeles.

Virus. The Armstrong CA 1371 strain of LCMV was used in these studies (1).

Antisera. The various antisera used to deplete different lymphocyte subpopulations are listed in Table 1. The following protocol was used for the antibody-plus-complement-mediated cell depletions. A total of 5×10^6 spleen or lymph node (LN) cells in 1.0 ml of medium were treated with antibody at an appropriate concentration for 1 h at 4°C. The cells were then washed and suspended in 1.0 ml of a 1:10 dilution of low-tox M rabbit complement purchased from Cedarlane. After 1 h of incubation at 37°C, the cells were centrifuged and suspended in 1.0 ml of medium. The dead cells formed a clump and were allowed to settle to the bottom of the tube, and the suspended cells were transferred to a new tube. The majority of the dead cells were removed by this procedure. A portion of the cell sample was stained with trypan blue and counted to determine the cytotoxic index, which was calculated as follows: cytotoxic index = $100 \times (\% \text{ cytotoxicity of antibody and complement} - \% \text{ cytotoxicity of complement alone}) / (100 - \% \text{ cytotoxicity of complement alone})$.

IC assay. BALB/c ByJ or WEH1 mice (6 to 12 weeks old) that had been infected at birth or in utero with LCMV were killed, and their spleen and LNs (inguinal, axillary, and cervical) were harvested. Single-cell suspensions of spleen and LN cells, free of erythrocytes, were prepared in RPMI 1640 medium supplemented with 10% heat-inactivated fetal

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TABLE 1. Characterization of spleen cells producing infectious virus

Antibody used (specificity) ^a	CI ^b	No. of LCMV ICs/10 ⁶ spleen cells ^c	% Reduction in LCMV ICs ^d
None		3,900	
None (complement only)		4,850	0
HO-13-4 (anti-Thy1.2)	32.4	2,100	46.1
C3PO (anti-Lyt1.2)	29.9	2,000	48.7
CG16 (anti-Lyt1.2)	40.4	375	90.3
Alloantiserum (anti-Lyt2.2)	11.5	4,250	0
AD4-15 (anti-Lyt2.2)	12.3	3,850	1.2
HO-2.2 (anti-Lyt2.2)	11.1	4,200	0
GK1.5 (anti-L3T4)	23.4	1,300	66.6
RL172.4 (anti-L3T4)	27.2	875	77.5
J11d (B cells)	44.6	4,450	0

^a Spleen cells pooled from four congenitally infected 8-week-old BALB/c ByJ mice were used in this experiment. AD4-15, CG16, and the alloantiserum against Lyt2.2 were purchased in the form of ascites fluids from Cedarlane and used at concentrations specified by the supplier. Hybridomas producing the monoclonal antibodies HO-13-4 (15), HO-2.2 (11, 20), J11d (2), and GK1.5 (6) were obtained from the American Type Culture Collection, Rockville, Md., and grown in our laboratory. Supernatants at a 1:2 dilution were used for the depletion experiments. Ascites fluid containing C3PO (14) was a gift from Mike Bevan, Scripps Clinic and Research Foundation, La Jolla, Calif., and was used at a 1:50 dilution. The hybridoma producing RL172.4 (5) was also provided by Mike Bevan, and its supernatant was used at a 1:5 dilution.

^b CI, Cytotoxic index.

^c IC assay was done as described in Materials and Methods and in the legend to Fig. 1.

^d Percent reduction in ICs was calculated by using 100% as the number of ICs produced by untreated cells.

calf serum, antibiotics, and L-glutamine. The mononuclear cells were washed extensively (at least three times) to remove free virus and then were assayed for infectious centers (ICs) by being plated on a confluent monolayer of Vero cells in 35-mm wells in six-well dishes (Costar, Cambridge, Mass.). After adsorption for 60 min at 37°C, the cells were overlaid with 3 ml of 0.5% SeaKem agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) in medium 199 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% heat-inactivated fetal calf serum, antibiotics, and L-glutamine. The plates were incubated for 4 days at 37°C and then overlaid with 2.0 ml of 0.5% agarose in medium 199 containing 0.01% neutral red (GIBCO). Plaques were scored the next day. To correct for plaques that may have been caused by residual free virus remaining after the three washes, the cell-free supernatant was also plated on Vero cell monolayers. Plaques produced by this supernatant (usually <10% of that produced by the cell fraction) were subtracted (from the number of plaques produced by the cells) to determine the number of ICs.

Immunofluorescence. For immunofluorescence assay, cytocentrifuge preparations of lymphocytes were fixed with acetone and stained with a polyclonal anti-LCMV guinea pig serum and then with fluorescein-conjugated rabbit anti-guinea pig immunoglobulin G (Cooper Biomedical Inc., Malvern Pa.).

Separation of lymphocyte subsets. To separate lymphocyte subsets inguinal and axillary LNs were removed from congenitally infected 6- to 8-week-old BALB/c ByJ mice, and single-cell suspensions were prepared from the pooled lymph nodes. Four to six mice were used in each experiment. The different cell populations were obtained as follows. (i) For adherent cells, the LN cells were treated with α Thy1.2 antibody plus complement to deplete the T cells and then were plated on plastic dishes for 4 h at 37°C. The

nonadherent cells were removed by being washed three times with medium and the adherent fraction was used for the IC assay. More than 90% of the adherent cells were macrophages as determined by esterase staining. (ii) For B cells, macrophages were removed by two cycles (2 h each at 37°C) of adherence on plastic dishes. The T and B cells were then separated by being panned on anti-kappa antibody-coated plates. The nonadherent fraction, consisting of T cells, was removed by being washed several times with medium. The plates were then incubated at 37°C for 90 min, and the adherent fraction, consisting of B cells, was recovered by vigorous pipetting. The resulting cell population consisted of >90% B cells with <5% contaminating T cells as determined by immunofluorescence with appropriate antisera. (iii) For L3T4⁺ T cells, macrophages were removed by two cycles of adherence on plastic dishes, and B cells were removed by two cycles of treatment with α J11d antibody plus complement. The enriched T-cell population was then treated twice with α Lyt2.2 (AD4-15) plus complement to remove Lyt2⁺ T cells. This procedure produced a cell population consisting of >90% L3T4⁺ cells. (iv) For Lyt2⁺ T cells, macrophages and B cells were removed as described above. The enriched T-cell population was then treated twice with α L3T4 (RL172.4) plus complement to deplete L3T4⁺ cells. The cell population thus obtained contained >90% Lyt2⁺ cells.

RESULTS AND DISCUSSION

Both neonatally and congenitally infected BALB/c ByJ and BALB/c WEH1 carrier mice were used in this study. The neonatally infected carriers were made by injecting 1-day-old mice intracerebrally with 10⁴ PFU of LCMV, and the congenitally infected carrier colony was derived from these neonatally infected mice. Spleen and lymph node cells from 6- to 12-week-old carrier mice were used to identify the subset of T cells infected with LCMV. Cells producing virus were scored by an IC assay on Vero cells. To determine the surface phenotype of infected cells, the spleen and LN cells 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 the spleen and LN cells is shown in Fig. 1. Treatment of spleen and LN cells with complement plus α Thy1.2, α Lyt1.2, or α L3T4 resulted in a decrease in the number of LCMV ICs. (The Thy1.2 and Lyt1.2 antigens are present on the cell surfaces of all T cells, whereas the L3T4 molecule is present on T helper cells only.) In contrast, depletion of Lyt2⁺ T cells had minimal or no effect on the number of cells producing virus. These results indicated that infection was primarily confined to L3T4⁺ Lyt2⁻ T cells with minimal involvement of Lyt2⁺ L3T4⁻ cells. Treatment of carrier mouse spleen and LN cells with α J11d plus complement also had a minimal effect on the number of LCMV ICs, indicating that few, if any, mature B cells were productively infected with virus.

To confirm the results shown in Fig. 1, three antisera against Lyt2.2 were used to deplete the Lyt2⁺ T cells. In addition, two antisera against Lyt1.2 and L3T4 were employed. The results of this experiment showing the cytotoxic index and the number of LCMV ICs after treatment of carrier spleen cells with the indicated antisera are presented in Table 1. Treatment with two monoclonal antibodies against L3T4 and two monoclonal antibodies against Lyt1.2 decreased the number of cells producing LCMV, whereas the three α Lyt2.2 antisera (two monoclonal antibodies and

an alloantisera) had minimal or no effect on LCMV-producing cells. This negative result was not due to ineffective depletion of Lyt2^+ T cells, since all three $\alpha\text{Lyt2.2}$ reagents used in this experiment killed between 11.1 and 12.3% of the spleen cells. This result was as expected since the spleen of an adult mouse contains 10 to 15% Lyt2^+ T cells. In addition, the efficacy of the $\alpha\text{Lyt2.2}$ antisera was checked by a functional assay; all three antisera abolished the killing activity of LCMV-specific CTL (data not shown).

The results showed that L3T4^+ T cells from LCMV carrier mice could form ICs on Vero cells (Fig. 1, Table 1). We next determined whether these T cells from carrier mice could produce infectious virus by themselves, i.e., without cocultivation with a sensitive cell line such as Vero. L3T4^+ T cells were purified from LNs of BALB/c ByJ carrier mice and cultured in 96-well plates (5×10^5 cells per well) at 37°C . At various times after culture (1, 16, 24, and 48 h), supernatants from triplicate samples were checked for the presence of infectious virus. At the same time, unfractionated LN cells were also checked for their ability to produce LCMV. The results of this experiment showed that (i) L3T4^+ T cells from carrier mice were capable of producing infectious virus and that (ii) the majority of the virus found in the superna-

FIG. 1. Surface phenotype of lymphocytes infected with LCMV during persistent infection in vivo. Mononuclear cells from spleen and LNs (inguinal, axillary, and cervical) of carrier mice were assayed for ICs as described in the text. Before the IC assay, the spleen and LN cells were treated with complement (C') alone or with the indicated antisera plus complement. The results are shown as percent reduction in ICs after treatment with the various antisera, with 100% as the number of ICs produced by untreated cells. The data presented are from 10 experiments. In each experiment, separate pools of spleen or LN cells from two to four carrier mice were used for the IC assay. Both congenitally and neonatally infected BALB/c WEH1 and BALB/c ByJ mice were used in these experiments; no differences were noted among these groups or between the spleen and LN samples.

FIG. 2. Production of infectious virus by L3T4^+ T cells from carrier mice. Purified L3T4^+ T cells and unfractionated LN cells were cultured in 96-well plates (5×10^5 cells per well) in RPMI 1640 medium containing 10% fetal calf serum and 5×10^{-5} M β -mercaptoethanol. The plates were incubated at 37°C in an atmosphere of 5% CO_2 in air. Triplicate samples were collected at the indicated times, and the amount of infectious virus in the cell-free supernatants was determined by plaque assay on Vero cells.

tants of unfractionated LN cells was probably made by L3T4^+ T cells (Fig. 2).

The IC assay is a sensitive technique but will detect only cells producing infectious virus. To identify the type and number of cells expressing viral proteins, spleen and LN cells from carrier mice were separated into various subpop-

TABLE 2. Percentages of T4^+ T cells and adherent cells of carrier mice infected with LCMV

Cell type ^a	% of cells infected with LCMV as determined by:	
	IC assay ^b	IF ^c
Unfractionated LN	0.72	6.60
L3T4^+ T	1.10	9.10
Lyt2^+ T	0.09	0.78
B	0.02	0.10
Adherent	2.50	ND

^a LN cells from carrier mice were separated as described in Materials and Methods.

^b IC assay was done as described in Materials and Methods except for the following modifications with the adherent cells: the Vero cells were plated on top of the adherent cells, incubated at 37°C for 4 h to form a monolayer, and then overlaid with agarose in medium 199.

^c Immunofluorescence staining for LCMV antigen was done as described in Materials and Methods. At least 1,000 cells were counted in each sample to determine the percentage of infected cells. IF, Immunofluorescence; ND, not done.

FIG. 3. Immunofluorescence staining demonstrating LCMV antigen in purified L3T4⁺ T cells (A) and absence of virus in Lyt2⁺ T cells (B). The T-cell subsets were purified from LNs of congenitally infected 6- to 8-week-old BALB/c ByJ mice and were tested by immunofluorescence assay as described in Materials and Methods.

ulations, and the number of infected cells was determined by both the IC assay and immunofluorescence. The results of one such experiment with LN cells from BALB/c ByJ carrier mice are shown in Table 2 and Fig. 3. The procedures used for obtaining purified populations of L3T4⁺ T cells, Lyt2⁺ T cells, B cells, and adherent cells are described in Materials and Methods. The data show that LCMV-infected cells are enriched in the L3T4⁺ T-cell population, whereas Lyt2⁺ T and B cells show a much lower proportion of infected cells compared with the unfractionated LN cells (87.5 to 98.5% reduction; Table 2). These results are in agreement with the data showing that infection is confined primarily to the T-helper subset, with minimal involvement of Lyt2⁺ T cells and B cells (Fig. 1; Table 1). Virus was also found in the

adherent cell fraction that consisted predominantly of macrophages. Infection of macrophages during persistent LCMV infection has been previously reported (16, 21–23).

An interesting result was that approximately 10-fold more cells were infected as determined by immunofluorescence compared with the number determined by the IC assay. This was a consistent and reproducible finding. In three additional immunofluorescence versus IC experiments, the percentages of L3T4⁺ T cells infected with LCMV as determined by assay were as follows: 12.2 versus 0.77, 4.5 versus 0.54, and 10.0 versus 0.67. These results suggest that a substantial number of infected cells were not producing infectious virus at a given time. It should be pointed out, though, that the lower number of infected cells detected by IC assay may be

a reflection of a low efficiency of plating of T cells on the Vero cell monolayer. However, this is unlikely to be the sole reason for the lower number of infected cells detected, since immobilization of T cells in proximity to the Vero cell monolayer by the addition of 0.5 ml of 0.5% agarose after adsorption did not result in an increase in the number of ICs. Thus, it appears that at least some L3T4⁺ T cells of carrier mice are nonproductively infected with LCMV. Similar observations have been reported by Welsh and Oldstone (23), who showed that adherent peritoneal cells from LCMV carrier mice contained a greater proportion of infected cells as determined by immunofluorescence compared with that determined by IC assay. These results, along with the data presented in this paper, suggest some type of regulation on the production of infectious virus in carrier mice.

This study establishes two points. The first and major point is that T cells of the helper subset are infected with LCMV during persistent infection *in vivo*. The second point of interest is that, at any given time, a substantial number of these infected T4⁺ cells are not producing infectious virus. It is tempting to speculate that this persistent infection may functionally inactivate the T helper cells that are required for the generation of LCMV-specific immune responses. Mice persistently infected with LCMV are deficient in generating LCMV-specific CTL and delayed-type-hypersensitivity responses and also make low levels of antibody against the virus (3, 13, 18). The mechanism of this suppression is not fully understood. It is possible that LCMV-specific immune responses are inhibited by a lack of appropriate T helper cells. In this context, it is worth noting that macrophages, the cells of the immune system that interact with T helper cells, are also persistently infected with LCMV.

Another viral infection in which both macrophages and T cells of the helper subset are infected is acquired immunodeficiency syndrome (8, 9, 12). Acquired immunodeficiency syndrome is characterized by a severe and generalized immune suppression (10). In contrast, the primary defect in LCMV carrier mice is in the generation of LCMV-specific immune responses. These carrier mice do not exhibit a generalized immunosuppression; although we have noted some abnormalities in their CTL responses to Pichinde virus, a related arenavirus. The LCMV carrier mice make normal T- and B-cell responses to most antigens (3, 13, 18; Ahmed and Butler, unpublished data). Since the observed suppression is reasonably virus specific, it is possible that the infected T cells may represent LCMV-specific T helper cells. It should be pointed out, however, that at present, we do not know the precise nature of the infected T cells, nor do we know the relative requirement(s) of T helper cells in the generation of anti-LCMV CTL and antibody responses. Our future studies will address these issues and will also examine the role of genetic variants of LCMV, selected from spleens of carrier mice, in infection of T helper cells and suppression of T-cell responses (1).

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