Infection of Lymphocytes by a Virus that Aborts Cytotoxic T Lymphocyte Activity and Establishes Persistent Infection

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Summary

For viruses to establish persistent infections in their hosts, they must possess some mechanism for evading clearance by the immune system. When inoculated into adult immunocompetent mice, wild-type lymphocytic choriomeningitis virus (LCMV ARM) induces a $CD8^+$-mediated cytotoxic T lymphocyte (CTL) response that clears the infection within 7–14 d ($CTL^+[P^-]$). By contrast, variant viruses isolated from lymphoid tissues of persistently infected mice fail to induce a CTL response and are thus able to establish a persistent infection in adult mice ($CTL^-[P^+]$). This report compares the interaction of $CTL^+(P^-)$ and $CTL^-(P^+)$ viruses with cells of the immune system. Both types of virus initially bind to 2–4% of $CD4^+$ and $CD8^+$ T lymphocytes and replicate within cells of both subsets. The replication of $CTL^-(P^+)$ and $CTL^+(P^-)$ viruses in lymphocytes in vivo is similar for the first 5 d after initiating infection. Thereafter, in mice infected with $CTL^-(P^+)$ variants, lymphocytes retain viral genetic information, and infectious virus can be recovered throughout the animals' lives. In contrast, when adult mice are infected with wild-type $CTL^+(P^-)$ LCMV ARM, virus is not recovered from lymphocytes for >7 d after infection. A $CD8^+$-mediated anti-LCMV CTL response is induced in such mice. Clearance of infected lymphocytes is produced by these LCMV-specific CTLs, as shown by their ability to lyse lymphocytes expressing LCMV determinants in vitro and the fact that depletion of $CD8^+$ lymphocytes before infection with $CTL^+(P^-)$ viruses results in levels of infected lymphocytes similar to those found in undepleted $CTL^-(P^+)$-infected mice. Hence, CTL-mediated lysis of T lymphocytes carrying infectious virus is a critical factor determining whether virus persists or the infection is terminated.

A number of diverse viruses, including EBV, cytomegalovirus, varicella-zoster virus, herpes simplex virus, rubella virus, measles virus, hepatitis B virus, human T cell leukemia viruses, and HIV, are known to cause persistent infections in man, and produce diseases of medical significance (1, 2). Establishment of persistent virus infection in vivo requires that the virus must persist within cells. To achieve this, a lytic virus life-cycle must change to a nonlytic one. In addition, the infected cells must survive in the host, hence the host immune system must be evaded (reviewed in references 3 and 4). To achieve the latter, viruses may: (*a*) avoid recognition by the immune system, either by downregulating their protein expression, as exemplified by herpes virus latency (5–7), and by decreased expression of viral proteins on the surface of measles virus–infected cells (8, 9); or by downregulating MHC expression as occurs with certain adenovirus subtypes (10–12) and with cytomegalovirus (13); and/or (*b*) directly or indirectly interfere with cells of the immune system so that they no longer mediate protection (reviewed in reference 14). Examples of such interference include infection of monocytes and $CD4^+$ T lymphocytes by HIV resulting in immunosuppression (15); infection of CTL and NK cells by human cytomegalovirus (HCMV)[1], which abrogates their ability to kill HCMV-infected targets (16); and measles virus infection of B lymphocytes and CTL, which aborts both humoral and cell-mediated responses to the virus (17–19).

To understand how viruses avoid immune surveillance and persist, we have studied mice infected with lymphocytic choriomeningitis virus (LCMV). The wild-type Armstrong strain of the virus (LCMV ARM) causes a lifelong persistent infection, during which viral materials remain in many tissues, in animals infected in utero or neonatally (20–22). However,

[1] *Abbreviations used in this paper:* CNS, central nervous system; HCMV, human cytomegalovirus; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein.

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when this virus is inoculated into adult immunocompetent mice, it induces a $CD8^+$-mediated CTL response that, depending on the dose and route of administration, either clears infectious virus and viral materials from the animal within 7–14 d or leads to central nervous system (CNS) immune pathology and death (20, 21, 23). Virus recovered from the brain, liver, or kidneys of persistently infected mice maintains the parental $CTL^+(P^-)$ phenotype when inoculated into normal adult mice. By contrast, LCMV variants isolated from lymphoid cells of persistently infected mice fail to induce an effective CTL response when inoculated intravenously into adult animals and thus establish persistent infection ($CTL^-[P^+]$) (24, 25).

Comparison of $CTL^+(P^-)$ and $CTL^-(P^+)$ virus isolates derived from LCMV ARM and revertants from the CTL^- to the CTL^+ phenotype, at the molecular level (26, 27) has shown that a single amino acid change from phenylalanine to leucine at position 260 of the LCMV glycoprotein GP-1 is associated with the difference in biological phenotype. Similar results were obtained by Matloubian et al. (28) using reassortant viruses. How this single amino acid change is associated with or mediates its effect is not known. However, it is clear that the defect in the antiviral CTL response occurs at the induction rather than the recognition level, because: (*a*) mapping of the LCMV domains recognized by virus-specific CTL in a number of different haplotypes has shown that GP-1 amino acid 260 is not contained within any of the known epitopes (29–31); (*b*) the $CTL^-(P^+)$ phenotype is not MHC haplotype dependent; (*c*) cells infected with $CTL^-(P^+)$ derivatives of LCMV ARM are recognized by LCMV-specific CTL from ARM-infected mice just as efficiently as target cells infected with $CTL^+(P^-)$ viruses (32); and (*d*) $CTL^-(P^+)$ viruses can induce CTL responses when administered at low doses or via the intraperitoneal route.

In this paper, we focus on the interaction of $CTL^+(P^-)$ and $CTL^-(P^+)$ viruses with cells of the immune system, addressing four questions. First, do $CTL^+(P^-)$ and $CTL^-(P^+)$ viruses bind to T cells equivalently? Second, are binding and subsequent virus replication limited to a particular T lymphocyte subset, or are both $CD4^+$ and $CD8^+$ lymphocytes involved? Third, are there differences in the progression of the infection of immune system cells in mice infected with $CTL^+(P^-)$ or $CTL^-(P^+)$ viruses, and fourth, if so, what is the mechanism(s) that underlies these differences? We show here that both types of virus bind with equal efficiency to lymphocytes of the $CD4^+$ and $CD8^+$ subsets, and that this is reflected in their in vivo tropism early after infection. The number of infected lymphocytes detected differs thereafter. This is shown to be a consequence of $CTL^+(P^-)$ virus inducing LCMV-specific CTL that remove infected lymphocytes, whereas $CTL^-(P^+)$ virus fails to induce a CTL response. Lifelong infection of lymphocytes, cells from which the $CTL^-(P^+)$ immunosuppressive variant can be isolated, occurs. The result is a failure to generate an effective CTL response, thereby establishing a milieu allowing continuous viral replication in many tissues and avoidance of immune clearance.

Materials and Methods

Virus. Parental LCMV ARM 53b stock ($CTL^+[P^-]$) was obtained from a triple plaque-purified clone subsequently passaged twice in BHK cells (33). Clone 13 (a $CTL^-[P^+]$ variant) was derived from spleen cells of an adult BALB/WEHI mouse in which a persistent infection had been initiated at birth with ARM 53b (24). Virus was recovered from lymphocytes by infectious center assay (34, 35). Plaque-purified clone 13 was passaged twice in BHK cells. The titers of virus stocks were determined by plaque assay on Vero cells (33).

Mice: Infection and In Vivo Depletion of T Cells. BALB/WEHI mice maintained in the closed breeding colony of the Research Institute of Scripps Clinic were used for all experiments. They were infected as adults (6–8 wk old) by intravenous inoculation of 2×10^6 PFU of virus. At this dose, ARM 53b–infected animals generate a virus-specific CTL response that clears the infection, whereas clone 13–infected animals fail to mount an effective CTL response and become persistently infected (24).

In some experiments, mice were depleted of $CD8^+$ T cells in vivo by treatment with a subset-specific rat mAb, YTS 169.4 (36). The antibody was partially purified from ascites by ammonium sulphate precipitation, dialyzed against PBS, and adjusted to a concentration of 10 mg/ml. T cell depletion was achieved by intravenous administration of 0.1 ml of antibody (at 10 mg/ml) on days −2 and +2 relative to the time of virus infection (day 0). This treatment results in elimination of 95–98% of $CD8^+$ T cells from the periphery (36).

Mononuclear Cell Preparation and Purification of Lymphocyte Subsets. PBMC were purified by Ficoll-Hypaque gradient centrifugation of mouse blood, as described (34, 35). Briefly, heparinized blood was diluted one in three in PBS, pH 7.2, layered onto an equal volume of Ficoll-Hypaque solution (Sigma Chemical Co., St. Louis, MO), and spun at 600 *g* for 20 min at room temperature. Cells at the interface were collected, washed in MEM containing 7% heat-inactivated FCS, and contaminating erythrocytes were removed by lysis with 0.83% ammonium chloride. After two washes, the number of viable cells was determined using trypan blue.

Spleens were dispersed by pressing through a wire grid, then the cells were washed and passed through 45–60-mm nylon mesh to remove aggregates. Single-cell suspensions of mononuclear cells were then purified on Ficoll-Hypaque gradients as described above.

To enrich T cells from the total mononuclear cell preparations, adherent cells and B cells were depleted by panning on anti-Ig-coated plastic Petri dishes using the method described by Wysocki and Sato (37). Briefly, goat anti–mouse IgG and IgM (Boehringer Mannheim Biochemicals, Indianapolis, IN) was diluted to 5 mg/ml in PBS. Plastic Petri dishes (100 × 15 mm) were coated by incubation with 10 ml of the anti-Ig solution for 40 min at room temperature. The plates were washed four times with PBS and once with PBS containing 1% FCS; then cells were added: $2–3 \times 10^7$ cells suspended in 3 ml of PBS containing 5% FCS per plate. The plates were incubated on a level surface at 4°C for 40 min, swirled gently to redistribute unattached cells, and then incubated for another 30 min. The T cell–enriched, nonadherent cell fraction was collected by swirling the dish and decanting the supernatant and then washing the plate once gently with 5–10 ml of PBS containing 1% FCS. In several experiments, >85–90% of cells obtained were $Thy\text{-}1^+$, with <10% Ig^+ cells.

$CD4^+$ and $CD8^+$ subpopulations were isolated from the T cell–enriched preparations by FACS® (Becton Dickinson & Co., Mountain View, CA) as described (35, 38). $CD4^+$ and $CD8^+$ subsets were marked using the subset-specific rat mAbs YTS 191.1

(anti-CD4) and YTS 169.4 (anti-CD8) (36). 10^7 lymphocytes were incubated with 100 μl of neat hybridoma supernatant at 4°C for 45 min. The cells were washed three times with MEM containing 5% FCS, and incubated for 30 min at 4°C with the second antibody: 100 μl of fluorescein-labeled $F(ab')_2$ mouse anti-rat IgG (Jackson Labs Inc., West Grove, PA) diluted to 20 mg/ml in PBS containing 5% FCS. After an additional three washes, flow cytometry was performed on a FACS IV® (Becton Dickinson & Co.). A minimum of 10^6 positive cells was collected from each sample. Cells were sorted so that the purity of each subset exceeded 99%. This was frequently achieved in one sort, but if not, a second sort with more stringent gates was used to ensure >99% purity.

Binding of LCMV to Lymphocytes. Binding of virus to cells was assayed using biotin-labeled virus as described (39, 40).

Briefly, LCMV was purified by polyethylene glycol precipitation followed by centrifugation at 35,000 rpm for 30 min on a discontinuous renografin gradient (20). Purified virus (1–2 mg protein/ml) was incubated with *N*-hydroxysuccinimidiobiotin diluted to 1 mg/ml in dimethylsulphoxide (39, 40). A 5:1 ratio of virus to biotin was used. After removing free biotin by dialysis against PBS, aliquots of biotinylated virus were diluted in RPMI containing 7% FCS, and either used directly or stored at −70°C until required. Virus infectivity was titered by plaque assay, and only biotinylated virus retaining >50% infectivity was used in experiments.

For the binding assay, varying amounts of biotinylated virus (0.5–30 mg) were added to single cell suspensions of 10^6 lymphocytes and incubated on ice. After 45 min at 4°C, cells were washed twice and avidin-PE was added. After another 30-min incubation on ice, cells were washed, and the maximal amount of virus-biotin bound was determined by FACS® analysis. Specificity of binding was demonstrated by the ability of unlabeled virus to block LCMV-biotin binding, and the lack of binding of LCMV-biotin complexes to two cell lines (RMA and RMAS), which LCMV ARM 53b is unable to infect.

In several experiments, two-color immunofluorescence was done, using avidin-PE to identify virus-biotin complexes, and T cell subset–specific rat mAbs and FITC-labeled anti–rat antibody to mark T cell subpopulations.

Infectious Center Assay. As described by Doyle and Oldstone (34), serial 10-fold dilutions of cells (from 10^6/well) were plated on semi-confluent layers of Vero cells grown in six-well plates (Falcon Labware, Oxnard, CA). After adsorption for 60 min at 37°C, 0.5% agarose was added. After a 6-d incubation at 37°C, the remaining cells on the plate were fixed with 10% formaldehyde in PBS and stained with crystal violet. The results were calculated as the number of infectious centers per 10^6 viable mononuclear cells plated. Freeze-thawing cells before plating on Vero cells failed to release infectious virus.

CTL Assay. Virus-specific CTL lysis was quantitated with a standard ^{51}Cr release assay, conditions for which are detailed elsewhere (41). Effector cells were lymphocytes prepared by Ficoll-Hypaque density gradient centrifugation from the spleens of BALB/WEHI mice infected 7 d earlier by intraperitoneal inoculation of 2×10^5 PFU of LCMV ARM. E/T ratios of 100:1, 50:1, and 25:1 were set up. The H-2^d-restricted, LCMV-specific CTL clone HD-8 was also used as an effector cell, at an E/T ratio of 5:1. This clone and the H-2^d clone HD-9 recognize the LCMV nucleoprotein (NP) epitope amino acids 119–127 (42). The fibroblast cell lines BALB Cl 7 (H-2^d) and MC57 (H-2^b), CTL clone HD-9 (H-2^d), and a mixed T lymphocyte population prepared from spleens of H-2^d or H-2^b mice were used as target cells. In some experiments, to enable uninfected target cells to be recognized by LCMV-specific CTL, the LCMV NP peptide amino acids 116–129 were used to coat the target cells. Peptide was added to the assay medium at a concentration of 200 ng/ml (42).

All samples were run in triplicate, and the assay time was 5 h. Results are expressed as the percent LCMV-specific lysis, calculated as: 100× (experimental release − spontaneous release)/(maximum release − spontaneous release). The variance among triplicate samples was <10%.

Results

$CTL^-(P^+)$ and $CTL^+(P^-)$ Viruses Bind Equivalently both to Unfractionated Mononuclear Cells and to Specific $CD4^+$ and $CD8^+$ T Lymphocyte Subsets. Initially, binding of the immunosuppressive viral variant LCMV ARM clone 13 ($CTL^-[P^+]$) and the wild-type parental ARM 53b ($CTL^+[P^-]$) virus to mononuclear cells obtained from the spleen and peripheral blood was analyzed. As seen in Fig. 1 *A*, the avidin-PE conjugate alone did not bind to mononuclear cells. However, addition of either $CTL^-(P^+)$ or $CTL^+(P^-)$ biotinylated virus before avidin-PE resulted in a significant and equivalent extent of binding between mononuclear cells and LCMV (Fig. 1, *B* and *C*), in six independent experiments.

Next, to determine whether $CTL^-(P^+)$ and $CTL^+(P^-)$ viruses bound specifically to $CD4^+$ and/or to $CD8^+$ lymphocyte subsets, mAbs directed against either $CD4^+$ (Fig. 1 *D*) or $CD8^+$ (Fig. 1 *G*) lymphocytes and a FITC fluorochrome were used to identify these two subsets in FACS®. Double immunofluorescence then marked the binding of virus to these lymphocyte subsets. As shown in Fig. 1, equivalent numbers of $CD4^+$ cells bound both viruses: in this experiment, 2.6% of $CD4^+$ cells bound $CTL^-(P^+)$ virus (Fig. 1 *F*) and 2.4% bound $CTL^+(P^-)$ virus (Fig. 1 *E*). In three additional experiments similar results were noted: a range of 2.6% ± 0.5 $CD4^+$ cells bound $CTL^-(P^+)$ virus, while 3.3% ± 0.6 of $CD4^+$ cells bound $CTL^+(P^-)$ virus. $CD8^+$ lymphocytes also bound equivalently to $CTL^-(P^+)$ (Fig. 1 *I*) and $CTL^+(P^-)$ (Fig. 1 *H*) viruses: in repeated experiments binding ranged from 0.7 to 3.4%.

Similar results were obtained regardless of whether the cells used were obtained from peripheral blood or spleens, or whether lymphocyte subsets were identified with PE and virus binding by avidin FITC or vice versa.

Progression of Productive Infection of Mononuclear Cells after Infection of Mice with $CTL^-(P^+)$ and $CTL^+(P^-)$ Viruses. Having ascertained that $CTL^-(P^+)$ and $CTL^+(P^-)$ viruses bind equivalently to total mononuclear cells and T lymphocytes of both the $CD4^+$ and $CD8^+$ subsets in vitro, we went on to investigate their ability to productively infect these cells. Because it proved difficult to infect $CD4^+$ or $CD8^+$ lymphocytes in vitro, viral replication was studied in vivo. Fig. 2 illustrates that both $CTL^-(P^+)$ and $CTL^+(P^-)$ viruses could replicate in mononuclear cells from the spleens and peripheral blood of mice infected intravenously as adults, as determined by infectious center assay.

$CTL^-(P^+)$ and $CTL^+(P^-)$ viruses infected similar numbers of mononuclear cells (from either spleen or peripheral blood) during the first 3 d after inoculation. The number

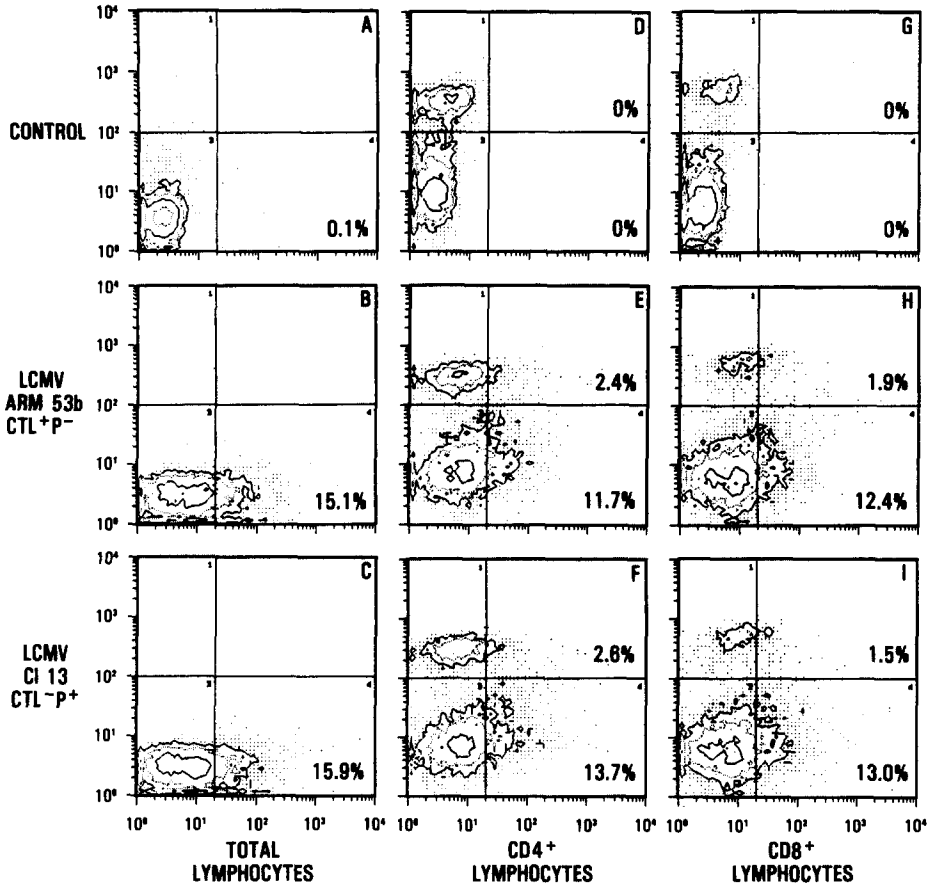

Figure 1. LCMV (both the parental virus ARM 53b [CTL$^+$(P$^-$)] and the clone 13 immunosuppressive variant [CTL$^-$(P$^+$)]) binds to mononuclear cells and to T lymphocytes of the CD4$^+$ and CD8$^+$ subsets. Binding is equivalent (percent binding given in the upper and/or lower right hand panels) for CTL$^+$(P$^-$) and CTL$^-$(P$^+$) viruses. Ficoll-purified lymphocytes from spleens of 8-wk-old BALB/W mice were incubated first with biotinylated LCMV and then with avidin-PE. Concurrently, lymphocyte subsets were marked with mAbs and FITC. Double-labeled cells were studied by FACS®. For each sort, a square divided into four segments is shown. The three sets of controls (*A*, *D*, and *G*) represent the mononuclear cell population and CD4$^+$ and CD8$^+$ subsets in the absence of biotinylated virus. (*B*, *E*, and *H*) binding of CTL$^+$(P$^-$) virus; (*C*, *F*, and *I*) binding of CTL$^-$(P$^+$) virus. Each upper left square records the CD4$^+$ or CD8$^+$ lymphocytes not binding biotinylated virus, and the upper right square depicts the CD4$^+$ or CD8$^+$ lymphocytes that do bind biotinylated virus. Results were similar in three additional experiments or when lymphocytes were obtained from peripheral blood.

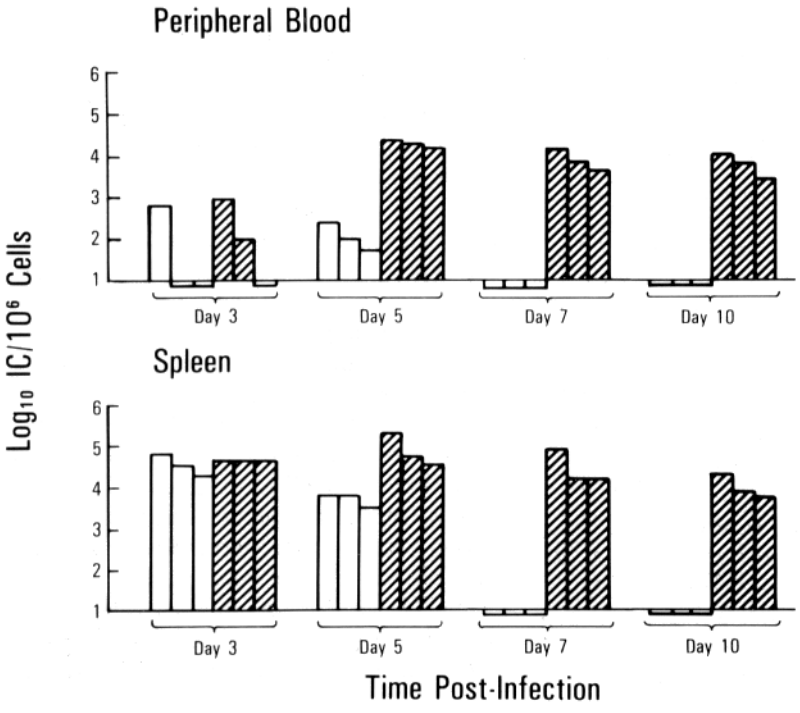

Figure 2. Infectious centers in total mononuclear cell populations from spleens and peripheral blood after infection of mice with CTL$^-$(P$^+$) or CTL$^+$(P$^-$) virus. Groups of 12 2-mo-old BALB/W mice were infected intravenously with 2 × 10^6 PFU of either clone 13 (▨), a CTL$^-$(P$^+$) immunosuppressive viral variant; or LCMV ARM 53b (□), the parental virus (CTL$^+$[P$^-$]) it was derived from. Three animals/group were killed at the indicated times after infection. Mononuclear cells were isolated from the peripheral blood and spleen of individual animals by Ficoll-Hypaque density gradient centrifugation. The number of infectious centers in each cell preparation was then determined. Results are expressed as $\log_{10}$ infectious centers/10^6 viable cells plated.

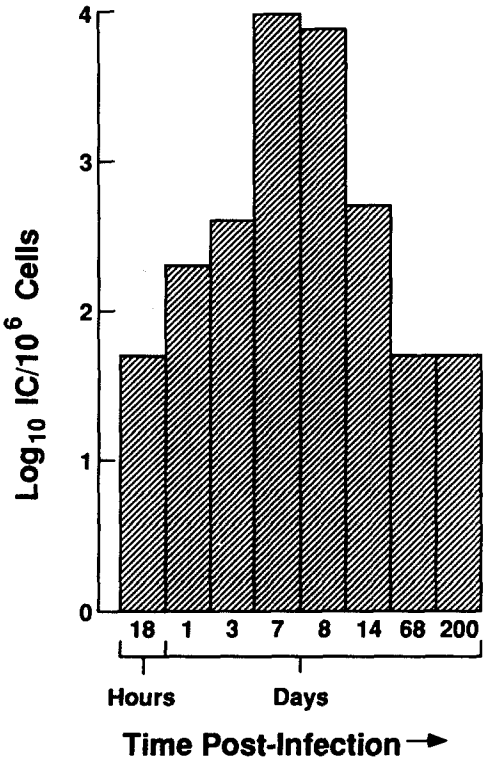

Figure 3. Kinetics of infectious centers in PBMC of mice infected with CTL$^-$(P$^+$) virus. BALB/W mice were infected intravenously with 2 × 10^6 PFU of LCMV clone 13 and, at the indicated times post-infection, four or more animals were bled and PBMC prepared by Ficoll-Hypaque density gradient centrifugation. The numbers of infectious centers were determined and the results expressed as $\log_{10}$ infectious centers/10^6 viable cells plated.

of infectious centers in CTL$^-$(P$^+$) infected mice remained relatively constant over the next 7 d, but in contrast, the number of infectious centers in mice infected with CTL$^+$(P$^-$) virus fell to an undetectable level by day 7 and thereafter (data shown up to day 10 only in Fig. 2).

Since the number of splenic mononuclear cells from CTL$^-$(P$^+$) virus–infected mice scoring as infectious centers remained high for the first 10 d after infection (Fig. 2), the kinetics of infectious centers were followed over a 200-d period. As shown in Fig. 3, the number of infectious centers increased for the first 7 d, peaking at day 7–8, and decreasing thereafter to $<10^2$ infectious centers/10^6 cells, a level that was maintained throughout the 200-d period examined.

CD4$^+$ and CD8$^+$ T Cell Subsets Are Infected In Vivo by both CTL$^-$(P$^+$) and CTL$^+$(P$^-$) Viruses. To look specifically at the infection of T lymphocytes of the CD4$^+$ and CD8$^+$ subpopulations, these cells were enriched to >99% purity from the spleens of infected animals using mAbs and FACS®, and analyzed by infectious center assays. Fig. 4 illustrates that T cells of both the CD4$^+$ and CD8$^+$ subpopulations replicated CTL$^-$(P$^+$) and CTL$^+$(P$^-$) virus. As observed with the total mononuclear cell population (Fig. 2), CD4$^+$ and CD8$^+$ T lymphocytes obtained from CTL$^+$(P$^-$)-infected mice scored as infectious centers only in the early phase (up to day 5) of infection, and became free from virus by 7 d after the initiation of infection. Again, in contrast, CTL$^-$(P$^+$)-infected mice failed to have cleared virus from their CD4$^+$ and CD8$^+$ T cells 7 d after infection.

A Specific Anti-LCMV CD8$^+$ CTL Response Generated after CTL$^+$(P$^-$) Virus Inoculation Causes the Decrease in Infectious Centers. 3 d after the inoculation of mice with either CTL$^-$(P$^+$) or CTL$^+$(P$^-$) viruses, similar numbers of both CD4$^+$ and CD8$^+$ T lymphocytes were infected, but a difference in the subsequent course of viral clearance (Figs. 2 and 4) was observed. The decrease in the level of infected lymphocytes in CTL$^+$(P$^-$)-infected animals could be due to either: (*a*) the infection being self-limiting, perhaps because only a subset of T cells are susceptible to infection, and once these have been lysed, no other lymphocytes are available for infection; or (*b*) virus-specific CD8$^+$ CTLs induced by CTL$^+$(P$^-$) virus clearing the infection by lysing lymphocytes expressing viral determinants.

To distinguish between these two possibilities, mice were depleted of CD8$^+$ T cells, infected with ARM 53b (CTL$^+$[P$^-$]), and the number of infectious centers in splenic mononuclear cells was compared with those in undepleted CTL$^+$(P$^-$)- and CTL$^-$(P$^+$)-infected mice. As shown in Fig.

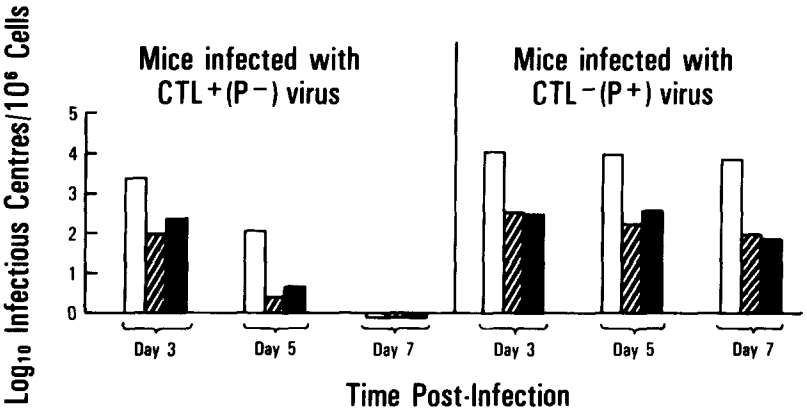

Figure 4. Infectious centers in CD4$^+$ and CD8$^+$ splenic T lymphocyte subpopulations from mice infected with CTL$^-$(P$^+$) or CTL$^+$(P$^-$) viruses. Groups of 12 BALB/W mice were infected intravenously with 2 × 10^6 PFU of virus and at the indicated times, four animals/group were killed and their spleens pooled. Spleen cell suspensions were prepared, and mononuclear cells isolated by Ficoll-Hypaque density gradient centrifugation. An aliquot of each cell suspension was kept on ice, and purified populations of CD4$^+$ and CD8$^+$ T lymphocytes were isolated from the remaining cells by panning on anti-Ig-coated plastic plates followed by FITC staining with subset-specific mAbs and FACS®. The CD4$^+$ and CD8$^+$ lymphocyte populations obtained were >99% pure by FACS® analysis. The number of infectious centers in the starting mononuclear cell preparation (□) and the CD4$^+$ (▨) and CD8$^+$ (■) selected populations were then determined, and are expressed as $\log_{10}$ infectious centers/10^6 viable cells plated.

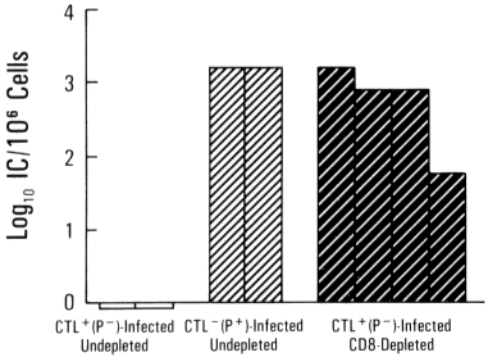

Figure 5. Infectious center levels in splenic mononuclear cells from CD8$^+$-depleted mice 7 d after infection with CTL$^+$(P$^-$) virus. Groups consisted of (*a*) BALB/W mice infected intravenously with 2 × 10^6 PFU of CTL$^+$(P$^-$) virus; (*b*) BALB/W mice similarly infected with CTL$^-$(P$^+$) virus; and (*c*) mice depleted of CD8$^+$ lymphocytes by two intravenous injections of a CD8$^+$-specific rat mAb, YTS 169.4, on days −2 and +2 relative to the day of infection (day 0), which were infected intravenously with 2 × 10^6 PFU of CTL$^+$(P$^-$) virus. 7 d after infection, splenic mononuclear cells were prepared from individual animals, and infectious centers assayed and expressed as $\log_{10}$ infectious centers/10^6 viable cells plated.

5, the CD8$^+$-depleted CTL$^+$(P$^-$)-infected mice had levels of infectious centers similar to those found in CTL$^-$(P$^+$)-infected animals, indicating that CD8$^+$ CTLs control the infection of lymphocytes in CTL$^+$(P$^-$) virally infected animals.

Final experiments established that LCMV-specific CTLs directly lysed lymphocytes expressing LCMV epitopes (Table 1). The LCMV-specific L^d-restricted CTL clone HD-8 recognizes the LCMV NP epitope 119–127, as do >96% of CTL generated in the spleen after a primary infection of H-2^d mice by LCMV. Both this CTL clone and a polyclonal population of primary CTLs lysed syngeneic (H-2^d) spleen T cells coated with the LCMV NP peptide. However, activated LCMV-specific CTL clones were resistant to CTL-mediated lysis.

Discussion

This paper compares the biology of a viral variant, CTL$^-$(P$^+$), that induces immunosuppression and allows the establishment of life-long persistence, with its parental virus, CTL$^+$(P$^-$), which generates the appropriate immune response required to terminate acute infection, clear virus, and thereby prevent persistence. The key molecular difference between the CTL$^+$(P$^-$) virus LCMV ARM 53b and its CTL$^-$(P$^+$) variant, LCMV clone 13, has recently been shown to be an amino acid change from phenylalanine to leucine at position 260 in GP-1 (27, 28). This mutation occurs close to the putative cleavage site where GP-1 and GP-2 are generated from the precursor GP-C (43, 44), suggesting that an event in processing may be important. However, how this amino acid change affects the CTL-inducing ability of the virus is unknown.

We have examined the interaction of these viruses with lymphocytes. CTL$^+$(P$^-$) virus binds to both CD4$^+$ and CD8$^+$ murine T lymphocytes, and both subsets are productively infected in vivo. Infectious progeny are made only during the first week after infection; by day 7 after infection, lymphocytes from such infected mice no longer score as infectious centers. This is because LCMV-specific CD8$^+$ CTL are generated after CTL$^+$(P$^-$) inoculation and these effector cells lyse lymphocytes expressing viral epitopes. The evidence

Table 1. *LCMV-specific CTL Lysis of Lymphocyte Targets*

Target cells	Effectors: Day 7 primary H-2^d CTLs (E/T ratio 100:1)	Effectors: H-2^d CTL clone HD-8 (E/T ratio 5:1)
LCMV-infected BALB Cl 7	97*	85
Uninfected BALB Cl 7	Nil	Nil
Uninfected BALB Cl 7 + 40 mg/well NP 116–129	90	50
Uninfected H-2^d spleen T cells + 40 mg/well NP 116–129	17	23
Uninfected H-2^b spleen T cells + 40 mg/well NP 116–129	Nil	Nil
H-2^d CTL clone HD-9	Nil	Nil
H-2^d CTL clone HD-9 + 40 mg/well NP 116–129	Nil	Nil

^{51}Cr release assay to assess the ability of LCMV-specific CTL to lyse lymphocyte targets expressing LCMV epitopes. LCMV ARM NP amino acids 116–129 contains the immunodominant H-2^d (L^d-restricted) epitope recognized by H-2^d BALB/c mice (42). This sequence is identical for CTL$^+$(P$^-$) and CTL$^-$(P$^+$) isolates of LCMV ARM viruses. Effector cells were either a splenic mononuclear cell preparation from H-2^d (BALB) mice primed 7 d previously with 2 × 10^6 PFU LCMV ARM 53b intravenously or a H-2^d CTL clone (HD-8) that specifically recognizes LCMV NP 116-129. ^{51}Cr release was measured after a 5-h lysis period; the results shown are the percent specific ^{51}Cr release: 100 × (sample release − spontaneous release)/(maximum release − spontaneous release).

* Percent specific ^{51}Cr released. Value reflects the mean of triplicate samples. Variance was <10%.

for this derives from experiments showing that CTL$^+$(P$^-$)-infected mice depleted of CD8$^+$-bearing lymphocytes fail to clear virus (Fig. 5) and that CD8$^+$ LCMV-specific CTL can lyse syngeneic lymphocytes expressing the appropriate viral epitope (Table 1). Thus, the clearance of lymphocytes containing virus occurs as a result of lysis by virus-specific MHC-restricted CD8$^+$ CTL rather than as a consequence of direct lysis by LCMV of mononuclear cells it infects. Hence, once CD8$^+$ effector cells are removed from virally infected mice, the numbers of LCMV-infected lymphoid cells are equivalent after infection with CTL$^+$(P$^-$) or CTL$^-$(P$^+$) viruses. This observation explains why wild-type virus does not persist in an immunocompetent host and confirms earlier observations of restricted replication of wild-type LCMV in lymphocytes during the first week after infection (34). The finding that infected lymphocytes are cleared in vivo as a result of lysis by virus-specific CD8$^+$ CTLs is of interest because of the previous assumption that T lymphocytes, in particular CD8$^+$ CTL, are resistant to CTL lysis (45, 46). Zalman et al. (47) described a homologous restriction factor within CTL believed to render them resistant to lysis by autologous CTL. Our results indicate that CTL can lyse CD4$^+$ or CD8$^+$ T cells carrying virus, and complement findings that CTL can lyse virus-infected B cells (48).

The CTL$^-$(P$^+$) immunosuppressive LCMV variant binds to and replicates in similar numbers of CD4$^+$ and CD8$^+$ lymphocytes for the first 5 d after infection as the CTL$^+$(P$^-$) parental virus (Figs. 1 and 4). However, in contrast to CTL$^+$(P$^-$) wild-type virus, by 7 d after the initiation of infection, CTL$^-$(P$^+$) virus aborts the generation of LCMV-specific CTL, thereby enabling virus to persist in lymphoid cells over the lifespan of the infected animal (Fig. 3).

Since LCMV ARM (both the CTL$^+$[P$^-$] parental strain and the CTL$^-$[P$^+$] immunosuppressive variant) binds to a small subset of both CD4$^+$ and CD8$^+$ lymphocytes in vitro, apparently only a small population of T cells must express a receptor for the virus. Our findings on the interaction of LCMV with T lymphocytes are reminiscent of those of McGrath and Weissman (49), showing that Moloney leukemia virus also binds to only a limited number of cells within the T cell population. The biochemical structure of the LCMV binding site on lymphocytes or other permissive cells is unknown, but is under active investigation. Although infection of murine lymphocytes has been repeatedly demonstrated in vivo in mice infected as newborns or in utero (34, 35, 50, 51), attempts to bind LCMV to or infect resting T cells in vitro were initially unsuccessful (50, 52). However, recent studies indicate that binding to and infection of resting and activated rodent lymphocytes can occur in vitro (53) (Borrow, P., E. Joly, and M.B.A. Oldstone, unpublished results). The explanation for these conflicting findings may come from a recent report (54) documenting the in vitro lymphoid tropism or lack of such tropism in a panel of recently isolated LCMV variants.

CTL$^+$(P$^-$) and CTL$^-$(P$^+$) LCMV isolates bind to ~2% of murine CD4$^+$ and CD8$^+$ T lymphocytes in vitro, and in vivo by day 3 permissively infect (as determined by infectious centers) ~0.05% of each subset. Thereafter, mice infected with CTL$^-$(P$^+$) virus show a peak in lymphoid infectious centers at 7–8 d, followed by a decline to lower levels that are maintained throughout the animals' lives (a 200-d period of observation). Although both CD4$^+$ and CD8$^+$ cells are equivalently infected during the first week after infection, by 28 d and thereafter, it is the CD4$^+$ lymphocytes that principally carry virus (35, 38, 51). Why and by what mechanism a selective decrease in the number of CD8$^+$ lymphocytes infected with LCMV occurs is not known. One possibility is that the balance of complete (infectious virus) to incomplete viral replication within the CD4$^+$ subset differs from that in the CD8$^+$ subset. A second possibility is that the accumulation of viral nucleic acid sequences over time is more toxic to or lytic in CD8$^+$ than CD4$^+$ T cells.

What then is the cause of the CTL-inducing property of CTL$^+$(P$^-$) virus and the inability to induce CTL of the immunosuppressive variant CTL$^-$(P$^+$) virus? The difference observed in the progression of infection of lymphocytes in CTL$^+$(P$^-$)- and CTL$^-$(P$^+$)-infected mice is a consequence of the virus's differing abilities to induce a CTL response and not a lytic property of the virus. The evidence for this conclusion is threefold. First, the LCMV-specific CTL response is CD8 mediated, and depletion of CD8$^+$ cells in vivo converts the kinetics of CTL$^+$(P$^-$) virus infection into those of CTL$^-$(P$^+$) infection. Second, CD8$^+$ LCMV-specific CTL recognize and lyse T cells expressing viral epitopes. Third, the replication of CTL$^+$(P$^-$) virus within lymphocytic cell lines in vitro is not associated with cell lysis, and replication of CTL$^+$ (P$^-$) virus in lymphocytes in vivo is not associated with virally induced lysis of the cells (Fig. 5). Further, the transient immunosuppression observed after infection of mice with various CTL$^+$(P$^-$) virus strains is also not thought to be a consequence of virus killing lymphocytes but instead may be due to disturbance of APC and/or perhaps the induction of suppressive lymphocyte responses (55–58).

In conclusion, a virus that causes persistent infection on the basis of immunosuppression would be expected to attack cells of the immune system and to abort the specific antiviral immune response(s). These principles have been documented here and elsewhere for LCMV infection (4, 25, 38, 51). Indeed, most if not all other viruses that cause persistence also infect lymphocytes and/or macrophages (reviewed in reference 14). As a consequence of infection, cells of the immune system may: (*a*) be lysed by the virus; (*b*) become targets of an immunopathological antiviral immune response; or (*c*) have their function impaired by persisting virus (cytopathology in the absence of cytolysis). Among the better studied examples are those of suppressed Ig synthesis or cytotoxic lymphocyte activity induced by measles virus infection of lymphocytes (17–19) and immunosuppression associated with HCMV (16). In nature, HCMV infects lymphocytes and monocyte/macrophages, and can persist in these cells in a latent form (59). Lymphocytes isolated from such naturally infected individuals can generate, in vitro, a HCMV-specific CTL response, except in the presence of recent clinical isolates of virus, which are immunosuppressive (16). CD4$^+$ T cells are infected in patients with AIDS, and such cells exhibit a defect in their ability to respond to soluble antigens

like tetanus toxoid (60). Similar to events with LCMV, measles virus, and HCMV infections, the HIV-infected lymphocytes showing immune dysfunction are apparently not lysed. Hence, information is gathering in many systems that viruses can infect lymphocytes and alter their functions. This lymphocyte-viral interaction may then explain the ability of the virus to persist and/or the presence of functional abnormalities associated with a particular viral infection. Thus, it may well prove profitable to evaluate diseases of unknown cause that involve defects in immune regulation for a viral etiology.

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