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Differential Immune Recognition of LCMV Nucleoprotein and Glycoprotein in Transgenic Mice Expressing LCMV cDNA Genes

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We have generated doubly transgenic (DT) mice that independently express cDNA genes for the nucleocapsid protein (NP) and the surface glycoproteins (GP) of lymphocytic choriomeningitis virus (LCMV). By RT-PCR, transcription of both transgenes was detected at low levels in brain and kidney but was not observed in the thymus. Additionally, transcription of the GP transgene was observed in the spleen. Following challenge with exogenous LCMV, an anti-NP CTL response was induced in LCMV-infected DT mice, suggesting that nonresponsiveness to NP had not been established. In contrast, LCMV-infected DT mice were nonresponsive to GP and failed to mount any CTL response against GP, either at Day 7 or Day 30 postinfection or following expansion of splenocyte populations *in vitro*. A significant number (33%) of adult DT mice survived intracerebral infection with LCMV, suggesting that virus-induced immunopathology in the central nervous system can be diminished by combined expression of the transgenes whereas no protective effect was conferred on singly transgenic mice, expressing NP or GP alone. The DT mice therefore create a novel host genetic background for comparative studies of the anti-LCMV immune responses relative to parental C57Bl/6 mice.

INTRODUCTION

Infections of mice with lymphocytic choriomeningitis virus (LCMV) have been studied for more than 60 years as reproducible and manipulable models for virus–host interactions (reviewed in Salvato, 1993; Asano and Ahmed, 1995; Southern, 1996). The LCMV model is particularly noted for rapid induction of potent anti-LCMV CTL responses that, according to the circumstances of infection, can cause lethal choriomeningitis or effectively clear infectious virus (Cole *et al.,* 1972; Byrne and Oldstone, 1984). In recent years, transgenic mouse technology (reviewed by Hanahan, 1989) has been used to express LCMV cDNA genes for the major structural proteins (NP, the internal nucleocapsid protein, or GP-C, the precursor for the mature surface glycoproteins) to determine whether the normal course of LCMV infection and/or host immune responses to LCMV infection are modified in the context of a genetically altered mouse. When the rat insulin promoter was used to target LCMV GP transgene expression in the β islet cells of the pancreas, the transgenic mice were either nonresponsive to GP (Oldstone *et al.,* 1991) or immunologically ignorant of GP (Ohashi *et al.,* 1991). However, in both systems, an anti-LCMV-specific CTL response could be induced in the transgenic mice, by exogenous LCMV infection, that resulted in cellular infiltration and destruction of the pancreatic islets (Oldstone *et al.,* 1991; Ohashi *et al.,* 1991).

All previous LCMV transgenic mouse studies have been performed with individual LCMV cDNA genes encoding either NP or GP-C. However, in order to look simultaneously at CTL responses to both NP and GP, we produced mice that express NP and GP-C transgenes (doubly transgenic, DT, mice) from independently regulated transcription units. By breeding the DT mice back onto the H-2^b background, we have contrived to ensure that defined immunodominant epitopes within both NP and GP-C are presented appropriately for potential recognition by anti-LCMV-specific CTLs (Whitton *et al.,* 1988b). In this report, we compare the outcome of acute LCMV infections and the character of the anti-LCMV CD8$^+$ T-cell response in adult DT mice and control C57Bl/6 mice.

MATERIALS AND METHODS

Cell lines

EL4. A thymoma cell line derived from C57Bl/6 mice (kindly provided by Dr. Steve Jameson, University of Minnesota).

MC57G. A methylcholanthrene-induced fibroblast cell line from C57Bl/6 mice. Both cell lines were propagated in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 m*M* L-glutamine, and antibiotics (RP10 medium).

Mice

C57Bl/6 mice were purchased from Charles River (Wilmington, MA) and were used at 6–12 weeks of age for breeding and as controls in experiments. Mice were in-

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fected with the Armstrong strain of LCMV (clone 53b; Dutko and Oldstone, 1983), either by intraperitoneal injection of 5×10^5 PFU or by intracerebral injection of 1×10^2 PFU.

Tissue preparation for histology

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and 3-μm sections were stained by the periodic acid-Schiff method (PAS).

Generation of transgenic mice

LCMV NP and GP-C singly transgenic mice were generated at Scripps Research Institute using standard transgenic procedures and reconstructed cDNA genes for NP and GP-C (Hogan *et al.,* 1986; Southern *et al.,* 1987; Oldstone *et al.,* 1991). Founder mice, shown to have integrated copies of the transgene, were crossed to C57Bl/6 mice to establish singly transgenic lines. The singly transgenic mice were cross-bred at the University of Minnesota to generate mice carrying both transgenes (DT mice) and DT mice were maintained by sequential matings with C57Bl/6 mice. The NP and GP-C transgenes were transmitted as unlinked Mendelian markers and the genotype of all progeny mice was established experimentally. NP and GP singly transgenic mice, produced at each DT $\times$ C57Bl/6 mating were used extensively as controls in these experiments.

Genotyping of transgenic mice

Tail DNA samples were screened by hybridization using LCMV-specific GP and NP probes (Southern *et al.,* 1987). Alternatively, screening was performed by PCR using DNA extracted from pieces of ear. The tissue was added to 20 μl of 50 m*M* Tris–HCl (pH 8.0), 2 m*M* NaCl, 10 m*M* EDTA, 1% SDS, and 1 μl of 20 mg/ml proteinase K (Boehringer-Mannheim, Indianapolis, IN) and incubated at 55°C for 15 min. After vigorous vortexing, the proteinase K digestion was repeated with an additional 20 μl of buffer. Sterile water was then added to a final volume of 200 μl and the mixture heated at 100°C for 5 min and cooled before direct addition to PCR amplification reactions.

RNA preparation

Freshly collected tissue was crushed between two frosted microscope slides and placed in guanidinium isothiocyanate (4.0 *M* guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1 *M* β-mercaptoethanol, and 1–2 drops antifoam; Chirgwin *et al.,* 1979). The cell lysates were layered over a 2-ml cushion of cesium chloride (5.7 *M* CsCl, 0.1 *M* EDTA) and centrifuged in a Beckman ultracentrifuge (SW41 swinging bucket rotor) at 30,000 rpm for 16 hr. RNA pellets were resuspended in sterile TE (10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA) and ethanol precipitated. mRNA was purified from total cell RNA using the PolyATtract mRNA magnetic particle isolation system (Promega, Madison, WI).

RT/PCR amplifications

Tissue mRNA samples were treated with 1 unit of amplification grade DNase I (Gibco BRL, Gaithersburg, MD) for 15 min at room temperature and then heated at 65°C for 10 min. The DNase I-treated mRNAs were mixed with 500 ng of random hexanucleotide primer, heated to 70°C for 10 min, and then annealed by slow cooling to room temperature. The annealed samples were incubated in a 40-μl reaction cocktail with each deoxyribonucleoside triphosphate at 0.25 m*M,* 25 units RNase inhibitor (Promega, Madison, WI), 0.1 *M* Tris–HCl (pH 8.3), 140 m*M* KCl, 10 m*M* $MgCl_2$, and 15 U of AMV reverse transcriptase (Boehringer Mannheim) for 1 hr at 42°C. The reactions were stopped by heating for 5 min in a boiling water bath and chilling on ice.

PCR amplification of cDNAs was performed in separate reactions containing 100 ng each of unique 5′ and 3′ oligodeoxynucleotide primers. The NP primers were NP2 (5′ GTG CAG AAG AAC TGA TGT TCT 3′) and NP8 (5′ AGA ACT GCC TTC AAG AGG TCC T 3′). The GP primers were GP1 (5′ CTC TAG ATC AAC TGG GTG TCA G 3′) and GP3 (5′ CAG CCA CTC CTC ATG TAT TTT CC 3′). Five microliters of each cDNA product was incubated with the primers and 1.5 units of Taq DNA polymerase (Boehringer Mannheim) in a buffer containing 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% gelatin, and each deoxyribonucleoside triphosphate at 50 μ*M.* The reaction mixtures were then amplified through 40 cycles at 60°C for 1 min, 72°C for 2 min, and 94°C for 1 min, followed by final incubations at 60°C for 1 min, and 72°C for 10 min. The PCR amplifications were carried out in an automated thermal cycler (Perkin–Elmer–Cetus, Emeryville, CA). Reaction products were analyzed by agarose gel electrophoresis then DNA within the gel was transferred to nylon membranes for hybridization with NP or GP-C region-specific probes (Southern *et al.,* 1987).

Splenocyte preparation

Spleens were aseptically removed and single cell suspensions were obtained by grinding the spleens between the frosted ends of microscope slides. Erythrocytes were lysed by 0.83% NH_4Cl treatment and intact cells were then pelleted and washed twice in medium to remove traces of NH_4Cl. LCMV-immune splenocytes were prepared from adult C57Bl/6 mice immunized 45 days previously with 10^5 PFU of LCMV-Arm and 1×10^6 cells were introduced into recipient mice by intraperitoneal injection.

Flow cytometry

Two-color staining was performed by incubating 1×10^6 splenocytes with anti-CD4 or anti-CD11b and anti-

CD8 for 30 min on ice. Directly conjugated antibodies were used: anti-CD4-PE (rat IgG2a), anti-CD8-FITC (rat IgG2a), and anti-CD11b-PE (rat IgG2b) (Pharmingen, San Diego, CA). Samples were analyzed on a Becton–Dickinson FACScan using Lysis II software.

Proliferation assays

Spleen cells were cultured at 1×10^5 cells per well in 96-well flat bottom plates in 200 μl RPMI (Biofluids Inc., Rockville, MD) with 10% FCS (Hyclone, Logan, UT) in a humidified incubator at 37°C with 5% CO_2. Cells were activated either with the T-cell mitogen concanavalin A (ConA, 0–10 μg/ml) or peptides (1–10 μM), corresponding to the immunodominant CTL epitopes recognized on the $H2^b$ background: NP 396–404 (Schulz *et al.,* 1989; Gairin *et al.,* 1995), GP 33-41 (Klavinskis *et al.,* 1990), and GP 276–286 (Oldstone *et al.,* 1988). Human recombinant IL-2 was added at 10 U/ml to control wells to enhance viability and stimulate proliferation for individual splenocyte preparations. Cells were pulsed on Day 2 of culture with 1 μCi/well [^{3}H]TdR (Dupont NEN, Boston, MA) and harvested the following day for liquid scintillation counting. All experimental combinations were performed in triplicate. Data were analyzed and converted to graphic form using Cricket Graph III (Computer Associates Software) for the Macintosh with vertical lines representing $\pm$ standard deviation from the mean.

CTL assays

Chromium release assays for CTL activity were performed largely as described previously (Byrne and Oldstone, 1984; Oldstone *et al.,* 1988; Whitton *et al.,* 1989). CTLs were induced by infecting mice intraperitoneally (i.p.) with 5×10^5 PFU of LCMV ARM and splenocytes were collected 4, 7, or 30 days later. To test CTL target specificities, 1×10^6 EL4 target cells were simultaneously incubated with ^{51}Cr sodium chromate (Dupont NEN, Boston, MA) and coated with 1 μM peptide in a humidified incubator at 37°C with 5% CO_2 for 1 hr. Cells were then washed 3$\times$ in RPMI/10% FCS and 1×10^4 target cells were plated with effector CTLs at E:T ratios of 50:1 and 25:1 in microtiter plate wells. An equal volume of complete RPMI 1640 was added in place of effector cells for measurement of ^{51}Cr spontaneous release, and 100 μl of 2% Triton X-100 was used to record maximum release. All samples were prepared in triplicate. After incubation for 6 hr at 37°C with 5% CO_2, the plates were centrifuged for 5 min at 3000 rpm, and 100-μl samples of supernatant were collected and counted on a Gamma 5500 instrument (Beckman Instruments, Inc., Palo Alto, CA). The percentage of specific ^{51}Cr release was calculated as follows: [(experimental cpm $-$ spontaneous release cpm)/(maximum release cpm $-$ spontaneous release cpm)] $\times$ 100.

RESULTS

Expression cassettes were constructed that placed the LCMV (Armstrong strain) NP cDNA gene adjacent to the mouse metallothionein I promoter (MT-NP, Glanville *et al.,* 1981; Fig. 1A) and the LCMV (Armstrong strain) GP-C cDNA gene adjacent to the SV40 early region promoter (SV-GP, Oldstone *et al.,* 1991; Fig. 1B) and DNA-positive founder mice were generated by microinjection and reimplantation of fertilized eggs (Hogan *et al.,* 1986). Crossbreeding between singly positive NP and GP-C transgenic mice created mice that carried both transgenes (DT mice; Fig. 1C) and the DT mice were subsequently bred by sequential matings with C57Bl/6 mice. The transgenes were transmitted as unlinked Mendelian markers and the genotype of all progeny mice was established by nucleic acid hybridization and/or PCR amplification.

Transcription of the NP transgene was detectable by RT-PCR in brain and kidney but not in spleen or thymus and, with the same cDNA preparations from the same adult DT mouse tissues, GP-C transcription was detectable in brain, kidney, and spleen but not in thymus (Fig. 2). Concurrent RT-PCR amplification of a mouse β-actin sequence was used to verify that equivalent amounts of tissue-specific cDNA had been used (Fig. 2). The tissue profile of transgene expression, as defined by RT-PCR, was confirmed by Northern blotting and, from this analysis, GP transcription was also detected in lung (data not shown). RT-PCR amplifications were also performed with intron-spanning primers, to amplify and sequence a fragment containing the splice junction at the 3′ end of the SV-GP-C mRNA to verify that splicing had occurred correctly (data not shown). However, the absence of an intron in the MT-NP transcription unit precluded the use of intron-spanning primers for independent verification of NP transcription.

We also attempted to document tissue sites of transgene expression using a protocol for syngeneic transfer of LCMV-immune splenocytes into transgenic recipient mice (Oldstone *et al.,* 1986; Oldstone and Southern, 1993). However, examination of multiple brain and spleen sections from several uninfected recipient transgenic mice failed to reveal extensive histopathological damage at Day 7 or Day 14 after the cell transfer. Occasional minor infiltrates were observed against a background of largely normal tissue in the kidneys of transgenic recipients (Figs. 3A and 3B), suggesting that the level of transgene expression and/or that the number of cells expressing the transgene was not sufficiently high to support large-scale recognition of LCMV determinants by the transferred cells. For comparison, extensive kidney lesions were readily detected in C57Bl/6 mice, persistently infected with LCMV (Figs. 3C and 3D), even in the absence of transferred cells, and virus-induced immune complex disease is characteristic of persistent LCMV infections in adult mice (Oldstone and Dixon, 1970;

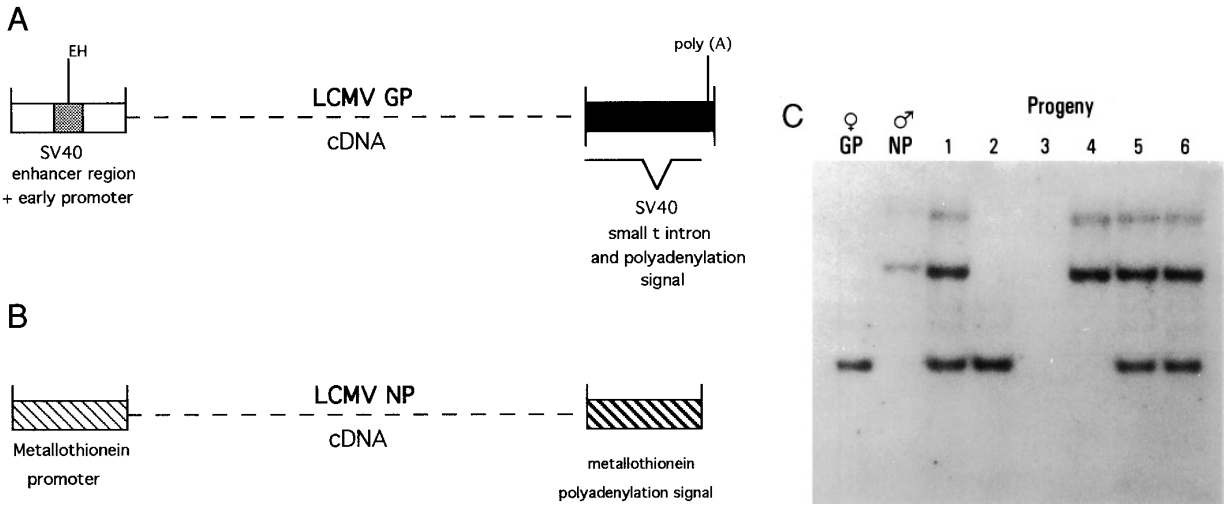

FIG. 1. Construction and characterization of NP and GP-C singly transgenic mice and cross-breeding to produce DT mice. (A) LCMV GP-C expression cassette containing the SV40 early region promoter, the SV40 small t intron and the SV40 early region polyadenylation site (Oldstone *et al.,* 1991). (B) LCMV NP expression cassette containing the mouse metallothionein I promoter and polyadenylation site (Glanville *et al.,* 1981). (C) Detection of LCMV cDNA genes in transgenic mice. Genomic DNA was extracted from a singly transgenic GP-C female mouse, a singly transgenic NP male mouse, and 6 progeny produced by mating these singly transgenic parents. DNA samples were digested with *Eco*RI and fractionated by electrophoresis in a 1% agarose gel. DNA bands within the gel were transferred to a nylon membrane for hybridization with a mixed probe containing both NP and GP-C labeled sequences. By this assay, progeny 1, 5, and 6 were identified as doubly transgenic (DT) mice, 2 was GP-C singly transgenic, 4 was NP singly transgenic, and 3 was nontransgenic.

Buchmeier and Oldstone, 1978). We have also noted the appearance of cellular infiltrates and mesangial proliferation within the glomeruli of kidneys from aged, unmanipulated transgenic mice (mice $>$ 1 year old, Figure 3E and F), but the underlying cause has yet to be defined.

Acute peripheral LCMV infection of normal adult mice induces a potent anti-LCMV CTL response and virus is rapidly cleared (Marker and Volkert, 1973; Moskophidis *et al.,* 1987). Paradoxically, the emergence of an anti-LCMV CTL response coincides with a generalized transient immune dysfunction (Jacobs and Cole, 1976), causing increased susceptibility to secondary infections (Mims and Wainwright, 1968). This transient immune dysfunction has been measured most effectively by loss of mitogen responsiveness when splenocytes from infected mice are stimulated *in vitro* (Saron *et al.,* 1990; Butz and Southern, 1994). As we began to search for changes induced by exogenous LCMV infection of DT mice, we examined the responsiveness of splenocytes from acutely infected DT mice to mitogenic stimulation *in vitro.* We found that there was a significant reduction in mitogen-driven proliferation for DT mice when splenocytes harvested from infected mice were compared with control, uninfected DT splenocytes, and this reduction exactly matched the findings for infected vs uninfected C57Bl/6 mice. This indicated that the general mechanisms for aberrant activation of splenic T cells, associated with dysregulation between IL-2 and IFN γ synthesis (Colle *et al.,* 1993; Ravzi and Welsh, 1993; Butz and Southern, 1994), were triggered in both LCMV-infected C57Bl/6 mice and LCMV-infected DT mice (Fig. 4). Splenic enlargement and expansion of the white pulp areas were found to be equivalent in DT mice and C57Bl/6 mice at Day 7 post-LCMV infection (data not shown) and FACS staining profiles with splenocytes from infected DT and C57Bl/6 mice were virtually indistinguishable. In particular, relative to uninfected controls, CD4:CD8 ratios were inverted after infection and there was a characteristic expansion of $CD11b^+$ cells in DT and C57Bl/6 mice (Table 1), as has been reported previously in acute peripheral LCMV infection of C57Bl/6 mice (McFarland *et al.,* 1992).

In previous studies with LCMV-transgenic mice, exogenous peripheral challenge with infectious LCMV has been reported to disrupt an immunologically nonresponsive state and anti-LCMV CTLs have been induced (Oldstone *et al.,* 1991; Ohashi *et al.,* 1991). In this study, we have exploited the possibility of immunological nonresponsiveness to both NP and GP-C in the DT mice to assess whether DT mice would be resistant to intracerebral (ic) challenge by LCMV. Infection of normal mice by ic injection with LCMV-Arm (Armstrong strain) results in an immune-mediated choriomeningitis and the majority ($>$90%) of the infected mice die 6–9 days postinfection (Rowe, 1954; Hotchin, 1962). When mice from the DT transgenic breeding colony were challenged by ic injection of LCMV-Arm, there was significant mortality for singly transgenic NP or GP-C mice and nontransgenic controls but 33% of the DT mice survived (Table 2). A lethal outcome for ic challenge of singly-transgenic mice (and nontransgenic controls) was entirely expected given that the anti-LCMV CTL response induced in $H2^b$ mice includes target epitopes contained within NP and GP-C

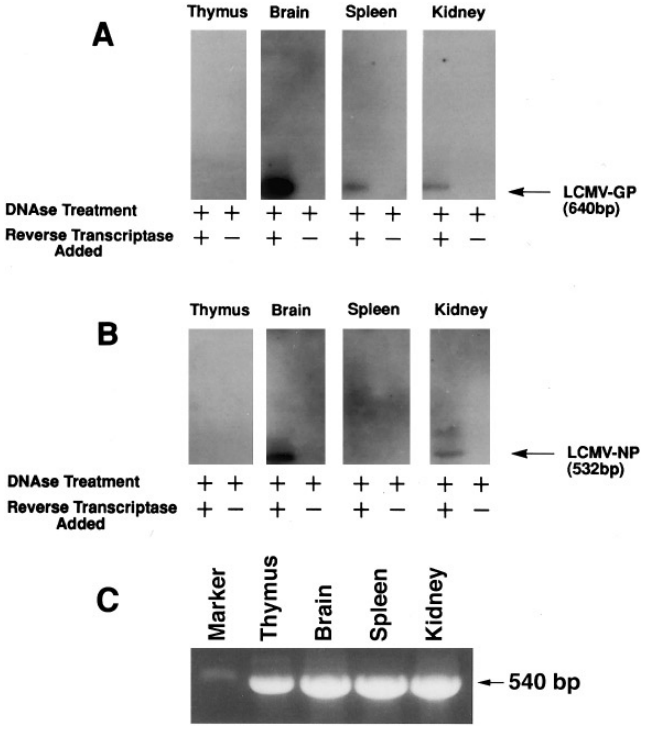

FIG. 2. Identification of tissue sites of expression of the LCMV NP or GP-C transgenes by RT-PCR amplification. Total cell RNA preparations (Chirgwin *et al.,* 1979) were obtained from organs of an adult mouse that had been treated with Zn^{2+} to induce metallothionein transcription (Palmiter *et al.,* 1982). Tissue mRNA samples were treated with RNase-free RQ1 DNase (GIBCO) to remove traces of contaminating DNA, prior to initiating reverse transcriptase reactions. PCR products were analyzed by agarose gel electrophoresis, transferred to a nylon membrane, and then hybridized with LCMV NP or GP-C region-specific probes (Southern *et al.,* 1987). Arrows indicate bands of the appropriate size to be consistent with the expected products. Note the lack of any detectable transgene expression in the thymus. Negative control lanes, where reverse transcriptase was deliberately omitted from the cDNA synthesis reactions, do not show any LCMV-specific product. This established that the observed PCR products were derived from RNA templates rather than from genomic DNA contamination of the RNA templates (von Herrath *et al.,* 1994). (C) Control PCR amplifications with β-actin primers to establish equivalence between cDNAs synthesized from the different tissue mRNA preparations.

and that recognition of peptide fragments derived from either protein is sufficient to trigger lethal choriomeningitis. The intermediate survival frequency (33%) for intracerebrally infected DT mice was determined to be significant by chi-square analysis with one degree of freedom and suggested that the combined expression of both NP and GP-C transgenes was causing some alteration in the LCMV-specific CTL response. By monitoring mice throughout the course of the acute intracerebral infection, DT mice that would eventually survive were observed to display symptoms of LCMV infection (ruffled fur, hunched posture, and inactivity at some point in the interval 5–9 days postinfection), but not to the same extent as mice that succumbed to LCMV infection (Hotchin, 1962). By 4–5 weeks post-LCMV infection, the surviving mice had regained an overtly normal appearance and had cleared infectious virus from the circulation (LCMV plaque assays with plasma samples, data not shown).

In an attempt to monitor T-cell activation directly in ic-infected mice, we performed FACs staining with splenocytes harvested from visibly sick mice, at Day 7 postinfection. The distribution of $CD4^+$, $CD8^+$, and $CD11b^+$ T-cells was essentially identical for ic-infected C57Bl/6 and DT mice. Interestingly, ic-infected mice showed only modest disturbance in CD4:CD8 ratios (CD4:CD8, 1.5:1 uninfected and 1:1.3 at Day 7 postinfection) and there were substantially fewer $CD11b^+$ T-cells relative to the ip-infected mice (8.2–9.3% $CD8^+$, $CD11b^+$ double-positive splenocytes in ic-infected mice compared with 22.9–29.8% $CD8^+$, $CD11b^+$ double-positive splenocytes in ip-infected mice, Table 1). It is not clear if the difference observed in the T-cell content of the spleen reflects altered kinetics of T-cell activation and expansion, as a consequence of the different doses and routes of infection, and/or altered distributions of activated lymphocytes in the infected mice (Allan *et al.,* 1987).

LCMV-specific CTL responses, induced by peripheral infection of adult DT mice, were identified using *in vitro* chromium release assays (Byrne and Oldstone, 1984). LCMV-infected targets ($H\text{-}2^b$, MC57 cells) were killed by splenocytes from infected DT mice in an MHC-restricted manner (data not shown), but the epitope specificity for CTL recognition could not be inferred from this experiment because epitopes from both NP and GP-C are presented on the surface of LCMV-infected MC57 cells. Other CTL mapping studies have used vaccinia virus recombinants to express individual LCMV cDNA genes, or carboxyl-terminal truncations of cDNA genes (Whitton *et al.,* 1988a, b; Schulz *et al.,* 1989), but, in order to examine the responses to the immunodominant NP and GP-C epitopes directly, we decided to examine CTL recognition of target cells ($H2^b$, EL4 cells) coated with synthetic peptides. Previous work has identified the precise sequences of peptides spanning the immunodominant CTL epitopes within NP and GP-C (Oldstone *et al.,* 1988; Schulz *et al.,* 1989; Klavinskis *et al.,* 1990; Gairin *et al.,* 1995) and shown, at least for $H2^b$, that there is no recognition of alternative NP or GP-C epitopes in primary acute infections of normal mice (von Herrath *et al.,* 1994). Splenocytes collected from individual acutely infected DT mice consistently lysed EL4 target cells coated with NP peptide (NP 396–404) but the same preparations of splenocytes failed to lyse EL4 target cells coated with GP peptides (GP 33–41 or GP 276–286) (Table 3). In contrast, splenocytes from infected control mice (C57Bl/6 mice or nontransgenic littermates) readily lysed NP- and GP-coated targets (Table 3). Note that lysis with splenocytes collected from acutely infected C57Bl/6 mice was reproducibly lower with GP 276–286 coated targets than with GP 33–41 coated targets. This may be explained by reduced stability for the GP 276–286 peptide/class I MHC complex, resulting in rapid dissociation of the peptide

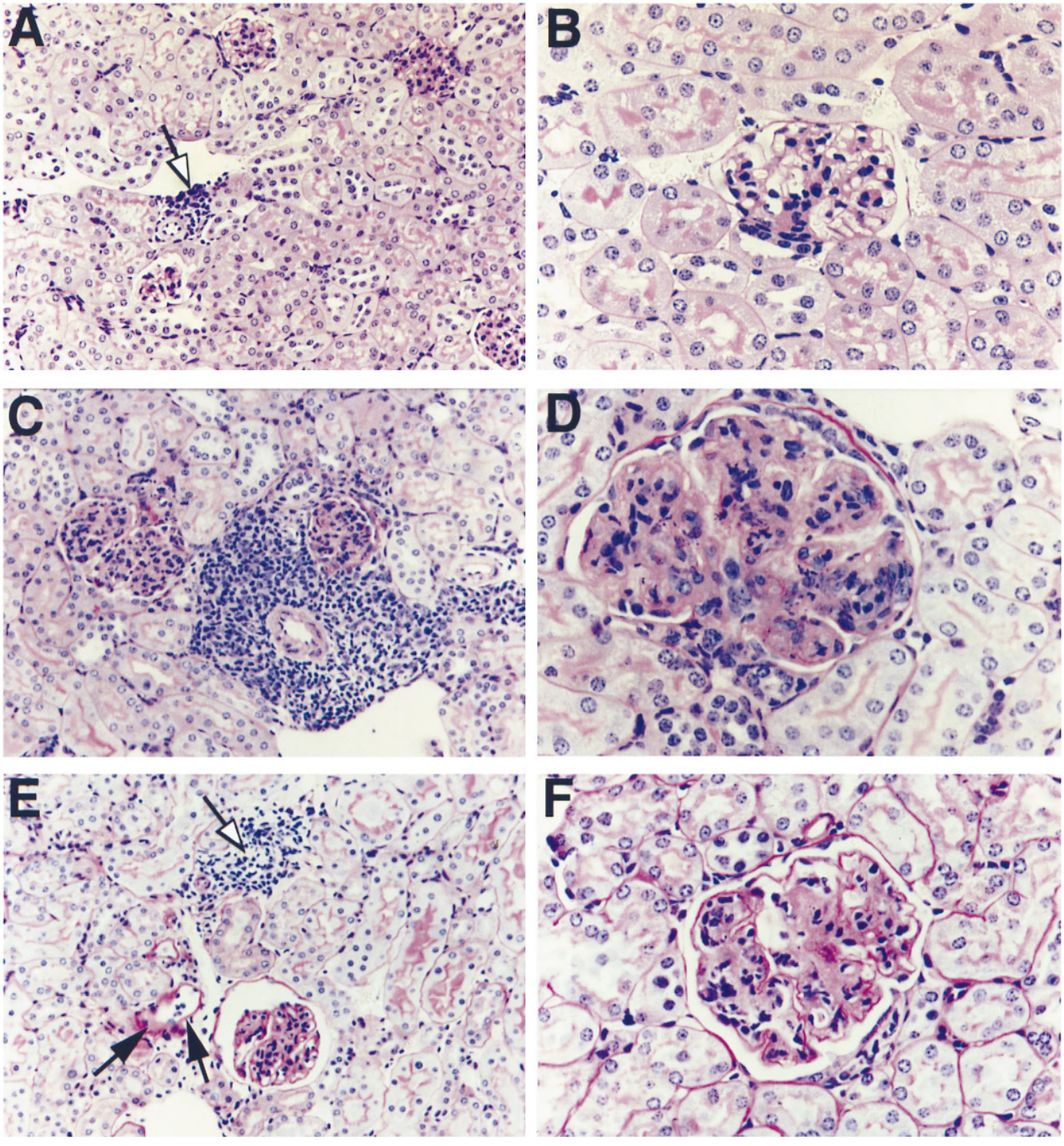

FIG. 3. Histological analysis of kidney sections from transgenic and persistently infected mice (periodic acid-Schiff staining, 3-μm sections). (A) NP transgenic mouse injected 7 days previously with LCMV immune splenocytes. There is a minor inflammatory cell infiltrate (white arrow) but the tubules and glomeruli appear normal. Similar infiltrates can be seen occasionally in C57Bl/6 controls (not shown) and in unmanipulated transgenic mice (E). Magnification $\times$200. (B) Glomerulus in a transgenic mouse injected 7 days previously with LCMV immune splenocytes. In this field, all elements are normal. Note the thin basement membrane, the patency of the capillary lumina, and low cellularity in comparison to D. Magnification $\times$400. (C) Adult C57Bl/6 mouse persistently infected with LCMV from birth. There is a substantial inflammatory cell infiltrate surrounding a small artery. The glomerulus on the left appears enlarged and both glomeruli in this field show hypercellularity. Magnification $\times$200. (D) Glomerulus in an adult C57Bl/6 mouse persistently infected with LCMV from birth. The glomerulus is enlarged and shows accentuation of lobularity. There is significant thickening of the capillary basement membranes and a distinct increase in cellularity due to mesangiocapillary proliferation and an inflammatory infiltrate. Note the prominence of the parietal epithelium, narrowing of the Bowman's space due to enlargement of capillary tufts, collapse of the capillary lumina, and karyorrhectic debris. Magnification $\times$400. (E) Unmanipulated aged DT mouse. There is a diffuse inflammatory cell infiltrate (white arrow) and a relatively normal glomerulus. An area of focal tubular atrophy is indicated by black arrows. Magnification $\times$200. (F) Unmanipulated aged DT mouse. This glomerulus is enlarged and shows some accentuation of lobular structure. There is hypercellularity in the lower left portion of the glomerulus, indicative of mesangial cell proliferation, and an increase in the mesangial matrix. Magnification $\times$400.

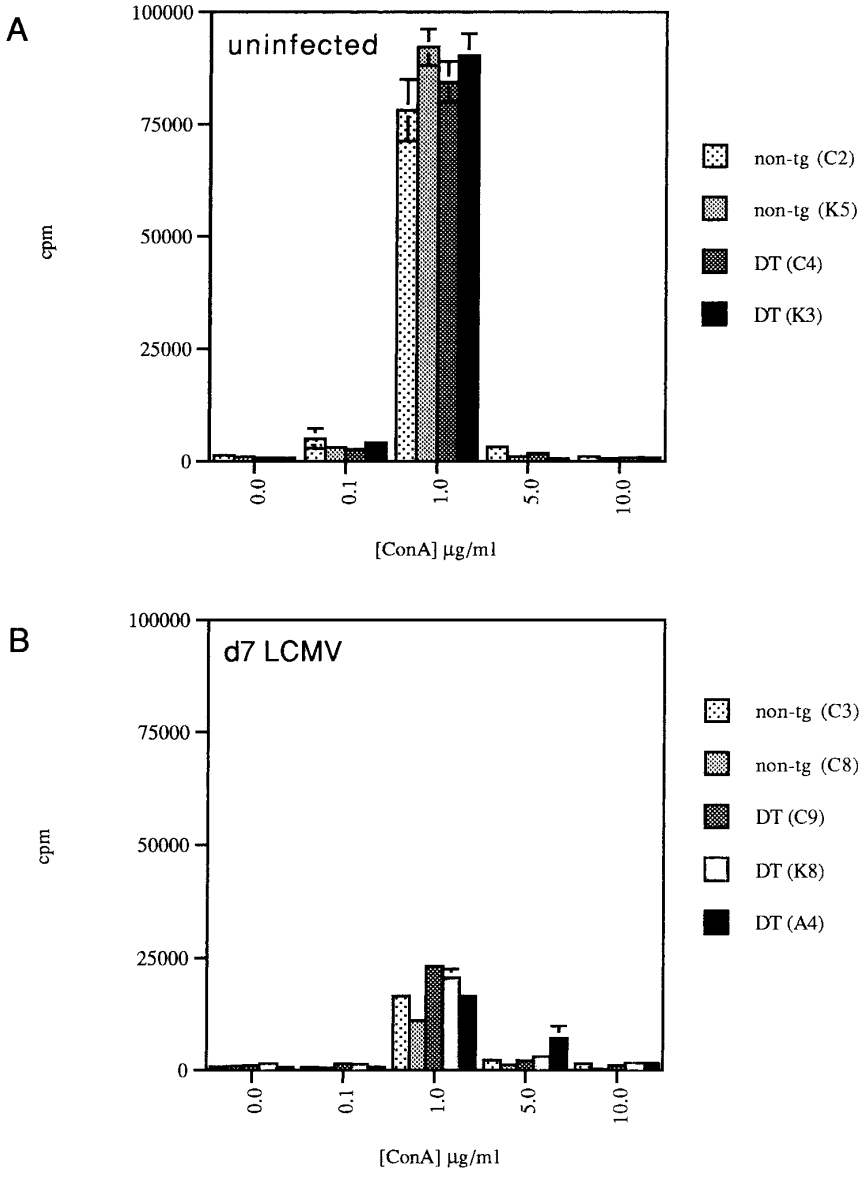

FIG. 4. Mitogenic activation *in vitro* with splenocytes from control and acutely infected mice. Splenocytes were prepared from uninfected mice (A) and mice infected 7 days previously with 1×10^5 PFU LCMV (Armstrong strain) (B) and were plated at 1×10^5 cells per well with increasing amounts of concanavalin A (ConA, Sigma). After 48 hr in culture, the cells were pulsed with 1 μCi/well [^{3}H]TdR, and harvested the following day for liquid scintillation counting (Butz and Southern, 1994). The results are shown for individual mice as the mean $\pm$ standard deviation for triplicate samples.

from the target cells and/or by lower primary CTL activity directed against the GP 276–286 epitope (Aichele *et al.*, 1994). The observation of differential target cell recognition by splenocytes from DT mice suggested that the NP and GP transgenes may be impacting differently on the immune responses in acute LCMV infection of DT mice. This interpretation was confirmed by examining *in vitro* proliferative responses to the NP peptide and GP peptides for bulk splenocytes, harvested 7 days after acute peripheral infection with LCMV. In the absence of exogenous IL-2, *in vitro* proliferation of DT splenocytes was stimulated by the NP 396–404 peptide (1 μM), but no proliferation above background levels was observed in response to either of the GP peptides (Table 4). The same differential proliferative response to NP and GP peptides *in vitro* was also found with splenocytes collected 7 days after ic-infection of DT mice (data not shown).

There are at least two distinct mechanisms that could account for the NP-specific CTL response in acutely infected DT mice: (a) the mice have been previously primed by NP and LCMV challenge is expanding a preexisting NP-specific CTL population or (b) the mice have remained immunologically ignorant of NP and LCMV challenge induces a conventional primary anti-NP CTL response. Preexisting anti-LCMV CTL populations have been documented by performing CTL assays with splenocytes harvested at Day 4 of acute LCMV infection, be-

TABLE 1

Flow Cytometric Analysis of Splenocytes Harvested from DT Mice 7 Days after Intraperitoneal LCMV Challenge

	Percentage of $CD4^+$	Percentage of $CD8^+$	CD4:CD8	Percentage of $CD11b^+$	Percentage of $CD11b^+$, $CD8^+$
LCMV i.p.					
DT (W6)	14.0	48.1	1:3.4	44.4	29.8
DT (W7)	18.9	38.6	1:2.0	38.2	22.9
C57Bl/6	21.9	45.7	1:2.1	39.0	24.7
Uninfected					
DT (X2)	16.9	11.3	1.5:1	10.6	1.3

Note. FACS analysis of splenocytes isolated from C57Bl/6 and DT mice 7 days after intraperitoneal infection with LCMV. Cell preparations were stained with directly conjugated antibodies: anti-CD4-PE, anti-CD11b-PE, and anti-CD8-FITC.

cause, at this time, there is insufficient CTL induction from a primary response to obtain effective *in vitro* killing (Zarozinski *et al.,* 1995). We were unable to demonstrate any CTL recognition above background levels for NP peptide-coated targets with splenocytes from DT mice at Day 4 of acute LCMV infection, suggesting that preexisting NP-specific CTLs were not present. Furthermore, CTL recognition of NP could not be uncovered by propagating Day 4 splenocytes *in vitro* with exogenous IL-2 (Table 5). Collectively these results indicate that the NP-specific CTL response in DT mice, observed at Day 7 of acute LCMV infection, reflects primary immune recognition of the immunodominant NP epitope.

We were also interested to assess whether, by analogy with the mice expressing LCMV transgenes in the pancreas (Oldstone *et al.,* 1991; Ohashi *et al.,* 1991, 1993), it would be possible to detect emergence of anti-LCMV CTL responses after recovery from acute peripheral infection. When splenocytes were collected at Day 30 postinfection, there was evidence for NP-specific CTL activity but there was variability in background levels of nonspecific killing in these assays (data not shown). However, when these same splenocyte preparations were cultured with IL-2 *in vitro* and then retested in CTL assays, there was convincing lysis of NP-coated targets and the continued absence of lysis with GP-coated targets (Table 5).

TABLE 2

Intracerebral LCMV Challenge of Transgenic Mice

Genotype					
NP	GP	Description	Number infected	Number surviving	Percentage of survival
+	+	DT	30	10	33
+	−	NP tg	33	4	12
−	+	GP tg	23	1	4
−	−	Non-tg	23	2	9

Note. Adult mice were infected by intracerebral injection of 100 PFU of LCMV ARM. A Chi-square analysis with 1 degree of freedom showed that 33% survival was statistically significant. Survival of C57Bl/6 mice after intracerebral infection with LCMV is less than 5%.

DISCUSSION

Expression of LCMV cDNA genes in transgenic mice creates a homologous system within which antitransgene immune responses can be assessed after exogenous virus challenge with LCMV. In previous studies (Oldstone *et al.,* 1991; Ohashi *et al.,* 1991, 1993), LCMV infection of transgenic mice expressing GP from the rat insulin promoter (RIP-GP) led to the induction of anti-LCMV CTL responses that, in turn, were responsible for extensive cellular infiltration and damage in the pancreatic islets. Thus, although transgene expression may contribute to an initial state of nonresponsiveness, the emergence of anti-LCMV-specific CTLs suggests that nonresponsiveness was overcome in the face of large antigen load and/or as a consequence of cytokine release during a potent immune response. There is general support for this latter mechanism because transgenic mice expressing both LCMV NP and γ IFN cDNA genes in the islets, developed anti-NP CTL reactivity spontaneously (Lee *et al.,* 1995).

Expression of an LCMV NP transgene in the thymus resulted in nonresponsiveness to the immunodominant CTL epitopes, because specific clones were eliminated by clonal deletion, but CTLs with reduced affinity for alternative epitopes were generated (von Herrath *et al.,* 1994). In experiments reported here, an MT-NP transgene did not appear to cause any alteration in anti-NP CTL responses following acute LCMV infection. Although the NP transgene was transcribed, the failure to induce nonresponsiveness may indicate that the levels and/or the sites of expression were inappropriate to cause immune alteration (Oehen *et al.,* 1994; Kundig *et al.,* 1995). In contrast, the SV-GP transgene did induce GP nonresponsiveness that was not disrupted by exogenous LCMV infection. Both NP and GP transgenes were expressed at similar low levels in brain and kidney and no expression could be detected in the thymus of adult DT mice, but the critical difference may be explained by GP expression in the spleen. In terms of priming an immune response in adult mice, direct splenic inoculation is known to be highly effective with low concentrations of antigen (Kundig *et al.,* 1995). Conversely, presentation of endogenous antigen in the spleen is capable of inducing

TABLE 3

CTL Responses to LCMV in LCMV Transgenic Mice

Source of Day 7 primary splenic CTL	E:T ratio	Specific ^{51}Cr released (%) from peptide coated EL4 targets: NP 396	GP 33	GP 276	L^d
Non-tg	50:1	78	71	24	7
	25:1	59	51	14	3
Non-tg	50:1	59	60	7	3
	25:1	36	45	2	3
Non-tg	50:1	51	53	5	2
	25:1	33	37	1	<1
DT (J4)	50:1	65	7	2	3
	25:1	44	2	3	2
DT (J6)	50:1	58	7	1	2
	25:1	37	0	0	2
DT (K1)	50:1	57	8	0	3
	25:1	35	7	0	2
DT (L2)	50:1	43	16	0	4
	25:1	25	6	0	2
DT (L3)	50:1	42	18	3	5
	25:1	16	5	0	2
NP tg (K6)	50:1	48	54	9	1
	25:1	23	27	3	<1
GP tg (K7)	50:1	58	9	0	2
	25:1	52	7	0	2
Uninfected					
DT	50:1	0	0	0	0
	25:1	0	0	0	0
Non-tg	50:1	0	0	0	0
	25:1	0	0	0	0
Gp tg (K3)	50:1	0	0	0	2
Np tg (K4)	50:1	0	0	0	8

Note. Splenocytes were harvested 7 days after i.p. infection with 1×10^5 PFU of LCMV. Effector cells at effector to target (E:T) ratios of 50:1 and 25:1 were used. EL4 target cells ($H2^b$ mouse thymoma cell line) were labeled with ^{51}Cr and pulsed for 1 hr with 1 μM of the indicated peptide. L^d is the synthetic peptide corresponding to the immunodominant region of LCMV NP recognized by $CD8^+$ CTLs in $H2^d$ mice and was used here as an irrelevant peptide control. Values represent the mean of triplicate determinations with variance less than 10%. Similar results were obtained with four additional infected DT mice.

peripheral nonresponsiveness (Webb *et al.,* 1990; Mondino *et al.,* 1996; Finkelman *et al.,* 1996) and this mechanism could account for the absence of GP-specific CTL responses in LCMV-infected DT mice. Further insight may be provided by revisiting the syngeneic immune cell transfer system to monitor the fate, both physically and functionally, of LCMV GP-specific T-cells/splenocytes that are transferred into DT recipients. Additionally, it would be of considerable interest to determine whether splenectomized DT mice retained their nonresponsiveness to GP-C following LCMV infection. We have attempted to identify the cellular site(s) of GP expression in the spleen using immunocytochemical staining of spleen sections or cytospin preparations of enriched dendritic cell populations but could not obtain consistent positive results (data not shown).

Intracerebral inoculation of LCMV into normal adult mice results in an immune-mediated choriomeningitis and >90% of the mice die 6–9 days after infection (Rowe, 1954; Hotchin, 1962). An intermediate survival frequency (33%, Table 2) for ic-infected adult DT mice was not anticipated and indicates a delicate balance between LCMV-induced immune activation triggering immunopathology or facilitating virus clearance from the CNS. LCMV-specific CTL recognize epitopes contained within both NP and GP-C on the $H2^b$ background in normal mice, whereas only a single immunodominant epitope in NP is recognized by $H2^d$ mice. Thus, in adult $H2^d$ mice, presentation of an NP epitope is seemingly sufficient to ensure a lethal outcome to ic-LCMV infection but the results in this study document 33% survival when anti-LCMV-specific CTL are targeted to NP in $H2^b$ DT mice. Collectively, these findings suggest that quantitative and/or qualitative differences may exist in the anti-NP CTL response in $H2^d$ and $H2^b$ mice. This comparison between $H2^d$ and $H2^b$ mice is unfortunately not exact because different NP peptides are presented by $H2^d$ and $H2^b$ mice but it is also possible that genetic differences between mouse strains may contribute toward the prospects for survival following LCMV ic challenge. Our data may also be interpreted to suggest that CTL induction and activity may be influenced by subtle variation within a given MHC background. According to the breeding program we have maintained, DT mice and GP-singly transgenic mice should be genetically identical except for the presence or absence of the NP transgene locus and both substrains of mice are fully nonresponsive to LCMV-GP. The results of the ic challenge experiments revealed that DT

TABLE 4

Stimulation Indices for Peptide Proliferation Assays

	Stimulation index: NP 396	GP 33	GP 276	IL2
Non-tg	6.3	7.0	5.7	330.4
DT (A1)	4.8	1.5	<1	212.5
DT (A3)	3.0	1.1	1.5	251.5
DT (B4)	4.5	1.3	1.5	274.7
Non-tg uninfected	<1	<1	<1	nd

Note. Splenocytes were harvested at Day 7 post-LCMV infection (i.p.), plated at 1×10^5 cells per well, and cultured with 1 μM LCMV peptide. Cultures were pulsed on Day 2 with 1 μCi/well [^{3}H]TdR, incubated overnight, and harvested for liquid scintillation counting. Stimulation index for proliferative response of individual mice to specific peptide: (avg cpm of splenocyte cultures from individual mice stimulated with the indicated LCMV peptide)/(avg cpm of duplicate splenocyte cultures stimulated with MHC mismatched peptide L^d). Stimulation index for proliferative response of individual mice to IL2: (avg cpm of splenocyte cultures from individual mice stimulated with 10U/ml IL2)/(avg cpm of duplicate splenocyte cultures stimulated with MHC-mismatched peptide L^d). Standard error was calculated from the mean of triplicate samples. Similar results were obtained with two additional i.p.-infected DT mice in an independent repetition of this experiment.

TABLE 5

CTL Responses to LCMV in LCMV Transgenic Mice

Source of Day 4 splenic CTL stimulated *in vitro* with 10U/ml IL2	Specific ^{51}Cr (%) released from peptide coated EL4 targets			
	E:T ratio	NP 396	GP 33	GP 276
C57Bl/6	50:1	8	8	<1
	25:1	4	2	<1
Non-tg (01)	50:1	4	8	<1
	25:1	7	<1	<1
DT (02)	50:1	7	0	<1
	25:1	4	2	<1
Np tg (04)	50:1	2	<1	<1
	25:1	1	0	<1
Gp tg (03)	50:1	3	2	13
	25:1	2	1	9
Source of Day 30 splenic CTL stimulated *in vitro* with 10 U/ml IL2	Specific ^{51}Cr (%) released from peptide coated EL4 targets			
	E:T ratio	NP 396	GP 33	GP 276
Gp tg (J1)	50:1	42	2	<1
	25:1	32	2	4
Gp tg (J5)	50:1	27	4	4
	25:1	26	2	9

Note. Splenocytes were harvested 4 or 30 days after i.p. infection with 1×10^5 PFU of LCMV and cells were cultured for 1 week in the presence of 10 U/ml IL2. Effector cells were used at effector to target (E:T) ratios of 50:1 and 25:1. EL4-target cells ($H2^b$ mouse thymoma cell line) were labeled with ^{51}Cr and pulsed for 1 hr with 1 μM of the indicated peptide. L^d is the synthetic peptide corresponding to the immunodominant region recognized by $CD8^+$ CTLs in $H2^d$ mice and was used as an irrelevant peptide control. Values represent the mean of triplicate determinations with variance less than 10%.

and GP-singly transgenic mice were distinguishable (33 vs 4% survival; Table 2) and implicate additional complexities, for example minor differences in virus replication and/or virus load, that can impact on immune activation and the overall outcome of intracerebral LCMV infection.

Partial protection from ic LCMV challenge (i.e., some surviving mice) was also achieved when LCMV-specific CTLs were induced by low antigen load (Battegay *et al.,* 1992; Whitton *et al.,* 1993; Bachman and Kundig, 1994; Yokoyama *et al.,* 1995; Zarozinski *et al.,* 1995). These results imply that there is a threshold concentration of antigen for efficient priming and induction of protective immune responses and that priming at, or below, threshold antigen levels may not confer consistent protection from subsequent exogenous virus challenge.

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