the yield) were then separated by SDS–polyacrylamide gel electrophoresis [10% gels (Novex, Encinitas, CA)]. The gel was stained with Coomassie blue, fixed, impregnated with Fluoro-Hance (Research Products International, Mount Prospect, IL), dried, and subjected to autoradiography.
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20. IL-8 was iodinated to a specific activity of 260 Ci/mmol as described (*15*). Single oocytes were incubated with ^{125}I-labeled IL-8 for 30 min on ice in 10 μl of binding buffer (Hanks balanced salt solution with 25 mM Hepes, 1% bovine serum albumin, pH 7.4). Unbound ligand was removed by centrifugation of the oocyte through 300 μl of F50 silicone fluid (General Electric, Waterford, NY). The tubes were quickly frozen and gamma emissions from the amputated tips were counted.
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22. Alignment with ten other G protein–coupled receptor sequences (*10, 11*) and examination of corresponding DNA sequences indicates that the apparent divergence of the IL-8 receptor from F3R between residues 92 and 105 is due to a frame shift in F3R.
23. Human genomic DNA (3 μg per lane) was digested with 6 units of Eco RI, Eco RV, Hind III, Pst I, or Xba I restriction endonucleases (Boehringer-Mannheim, Indianapolis, IN) and was then fractionated by electrophoresis on an agarose gel (1%). After denaturation in alkaline solution the DNA was transferred to a Nytran filter by capillary action.
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Viral Persistence in Neurons Explained by Lack of Major Histocompatibility Class I Expression

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Viruses frequently persist in neurons, suggesting that these cells can evade immune surveillance. In a mouse model, 5 × 10⁶ cytotoxic T lymphocytes (CTLs), specific for lymphocytic choriomeningitis virus (LCMV), did not lyse infected neurons or cause immunopathologic injury. In contrast, intracerebral injection of less than 10^3 CTL caused disease and death when viral antigens were expressed on leptomeningial and choroid plexus cells of the nervous system. The neuronal cell line OBL21 expresses little or no major histocompatibility (MHC) class I surface glycoproteins and when infected with LCMV, resisted lysis by virus-specific CTLs. Expression of MHC heavy chain messenger RNA was limited, but β_2-microglobulin messenger RNA and protein was made normally. OBL21 cells were made sensitive to CTL lysis by transfection with a fusion gene encoding another MHC class I molecule. Hence, neuronal cells probably evade immune surveillance by failing to express MHC class I molecules.

NEURONS ARE ESSENTIAL FOR RECEIVING, integrating, and passing information. Although they maintain many essential functions of an organism, they cannot be replaced once destroyed. Hence, it is likely that neurons have unique strategies to avoid injury. Neurons can be persistently infected by many viruses (*1*). The cardinal host response to eliminate virally infected cells is the generation of CTLs (*2*), which recognize and kill infected cells when viral peptides appear on the surface that are complexed to glycoproteins of the class I MHC (*3*). Yet, although activated CTLs can cross the blood brain barrier (*4*), they seem unable to lyse neurons that are persistently infected with any of several different RNA or DNA viruses.

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Neurons may have evolved a selective survival mechanism to avoid CTL recognition and lysis, such as a failure to present viral peptides complexed by MHC glycoproteins on the cell surface. Although the consequence of escape from CTL-mediated lysis would be favorable to the neuron, it would allow viruses to persist in these cells.

Lymphocytic choriomeningitis virus (LCMV) infection is controlled primarily by virus specific CTLs (*2, 5*). We generated CTL clones to the major epitopes of LCMV (*6*): CTL clones 228 and 232 are D^b-restricted and recognize amino acid residues (aa) 278 to 286 of the LCMV glycoprotein (GP); CTL clones HD8, HD9, and HD47 are L^d-restricted and recognize aa 119 to 127 of LCMV nucleoprotein (NP); and CTL clone Q9 is H-2^q–restricted and sees aa 116 to 127 of NP (*6*). CTL clone K39 is H-2^k–restricted and recognizes a non-GP, non-NP epitope located on the L RNA strand of the virus [either the viral polymerase or Z protein (*6*)]. During acute LCMV infection of the brain, virus replication is restricted primarily to cells in the leptomeninges and choroid plexus and rarely, if ever, in neurons [(*7*) and Fig. 1, top]. During persistent infection, the opposite occurs: neurons are heavily infected, but few other nervous system cells express viral antigens (Fig. 1, bottom). We took advantage of such observations to transfer LCMV-specific CTL intracerebrally to mice either acutely (87 mice) or persistently (36 mice) infected with virus. Virally infected neurons evaded CTL-mediated injury, and persistently infected mice remained alive throughout the 2- to 4-week observation period, despite receiving doses of CTLs $>1 \times 10^6$ per mouse. In contrast, intracerebral transfer of similar CTL clones to acutely infected mice resulted in immunopathologic injury and death within 7 to 12 days. This phenomenon was MHC-restricted since CTL clones were effective only when injected into mice of matched MHC haplotypes, including H-2^b, H-2^d, H-2^k, and H-2^q (Fig. 1). Death correlated directly with the number of CTL transferred. The 50% lethal dose (LD_{50}) end point ranged from 2×10^2 to 8×10^2 CTL for all haplotypes (tenfold dilutions of CTL from 10^6 to 10^1, six mice per group). Thus, although CTLs efficiently lysed virus-infected cells of the choroid plexus and leptomeninges, which express MHC class I glycoproteins, they did not kill neurons infected with virus.

To examine the mechanism by which neurons escape CTL lysis, we studied interactions between neurons and CTL in vitro. The OBL21 cell line was established by in vitro transformation of olfactory bulb cells of newborn CD1 mice (H-2^q haplotype) with a retroviral vector (*8*). Such cells re-

tained neuronal characteristics, including the expression of neurofilaments, neuron-specific enolase, and voltage-dependent potassium channels, and were permissive to LCMV infection. LCMV-infected, OBL21 cells were not lysed by MHC-restricted anti-LCMV CTLs, but H-2^q (SWR/J) fibroblasts were killed effectively (Table 1). Yet, these neuronal cells assembled and replicated infectious virus as determined by plaque titration of their supernatant fluids on susceptible Vero cells. The OBL21 cells, whether infected or not, expressed little MHC glycoproteins on their surfaces (Table 1). Hence, the inability of OBL21 neuronal cells to express adequate levels of MHC class I molecules may enable their escape from lysis by CTL.

Fig. 1. Expression of LCMV antigens in the brain during acute (top) and persistent (bottom) LCMV infections. In the acute infection, LCMV antigens were expressed in the leptomeninges, but not in neurons (arrow). Expression of MHC class I glycoproteins was restricted to infiltrating lymphocytes, endothelial cells, choroid plexus, and leptomeninges. In persistent infection, LCMV was found primarily in neurons. Immunofluorescence was done on 4 to 5 μM cryomicrotome sections, fixed with ether alcohol and 95% alcohol, then stained with a fluorescein-conjugated monoclonal antibody to LCMV or MHC molecules (*5*, *18*). Viral titers (*5*) in the central nervous systems ranged from 10^5 to 10^7 plaque-forming units per gram of brain in acutely infected and from $10^{4.5}$ to 10^6 plaque-forming units per gram of brain in persistently infected mice. When tenfold serial dilutions of cloned CTL were transferred (1×10^6 to 10) into haplotype matched acutely infected mice the LD_{50} dose varied from 2×10^2 to 8×10^2 CTL. During acute or persistent infection, adoptive transfer of these CTL into syngenic recipients lysed hepatic cells, whereas no evidence for neuronal lysis was noted. Initiation of acute and persistent infections and strategy for adoptive transfer of CTL are detailed elsewhere (*5*, *17*).

CTL transferred			In vivo target mortality			
Clones	H-2	LCMV epitope	H-2^b	H-2^d	H-2^k	H-2^q
Acute LCMV infection—leptomeninges and choroid plexus tropism						
228	D^b	GP2 278-286	6/6	0/6	ND	ND
32	D^b	GP2 278-286	5/5	0/5	ND	ND
HD8	L^d	NP 119-127	0/10	8/8	ND	ND
HD9	L^d	NP 119-127	0/8	7/7	ND	ND
HD47	L^d	NP 119-127	0/5	5/5	ND	ND
K39	H-2^k	L RNA	0/8	ND	8/9	ND
Q9	H-2^q	NP	0/5	ND	ND	5/5
Persistent LCMV infection—neuron tropism						
228	D^b	GP2 278-286	0/10	ND	ND	ND
32	D^b	GP2 278-286	0/10	ND	ND	ND
HD8	L^d	NP 119-127	0/4	ND	ND	ND
HD9	L^d	NP 119-127	0/3	ND	ND	ND
HD47	L^d	NP 119-127	0/5	ND	ND	ND
K39	H-2^k	L RNA	ND	ND	0/5	ND
Q9	H-2^q	NP	ND	ND	ND	0/4

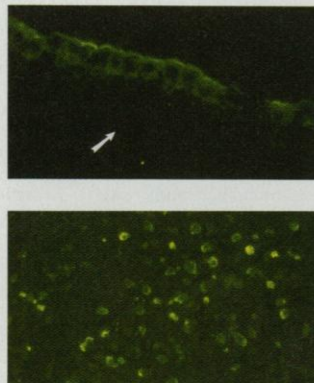

Table 1. OBL21 neuronal cells resist CTL killing. On the day of the assay, cells grown to near confluence were harvested and divided into two aliquots. One component was used to measure the surface expression of MHC class I molecules by immunofluorescence and flow cytofluorimetry (results shown in the upper part of the table). The second component was labeled with ^{51}Cr and used in a CTL assay. CTL results are shown in the lower part of the table. Surface expression of MHC class I molecules were determined by flow cytofluorimetry after incubation with MAbs 30-5-7 (to L^q and D^q) and 34-4-20 (to K^q) (*18*). Results shown are means of specific fluorescence index from analysis of 10^4 cells. [Negative values obtained from labeling cells with fluorescein isothiocyanate-conjugated (FITC) goat antibodies to mouse IgG were subtracted from the values obtained when the cells were incubated with primary antibody and FITC reagent.] Dead cells, stained with propidium iodide, were discounted. Concentrations of antibodies were saturating. Results shown from the CTL assay are the mean value of percent ^{51}Cr released from triplicate samples measured by a standard CTL assay (*6*). CTLs used were obtained from spleens of SWR/J mice that had been infected 7 days earlier with 2×10^5 PFU of LCMV. Effector (E) to target (T) ratio was the ratio of the number of CTLs to ^{51}Cr labeled target cells. Target cells were SWR/J fibroblasts, OBL21 neuronal cells, or IFN-γ–treated (20 IU/ml, 48 hours) OBL 21 cells. Variance among triplicates was <10%. Similar results were obtained from three independent experiments.

Condition	SWR/J fibroblasts treatment			OBL21 treatment			OBL21 + IFN-γ treatment		
	None*	Peptide†	LCMV‡	None*	Peptide†	LCMV‡	None*	Peptide†	LCMV‡
Flow cytometry (relative fluorescence)									
MAb 30-5-7	95	ND	115	7	6	10	260	250	350
MAb 34-4-20	65	ND	80	0	0	0	73	70	90
CTL assay (% ^{51}Cr released)									
E:T 100:1	5	47	70	7	4	4	4	14	35
E:T 50:1	3	29	40	3	2	2	2	6	17
E:T 25:1	3	20	24	0	0	2	0	2	8

*Untreated cells refer to cells not infected with virus and handled similarly. †Chemically synthesized peptide representing aa 116 to 129 (20 μg/ml) was used to coat uninfected cells for the CTL assay. Peptide was added to cells that were then cultured overnight and used in the CTL assay. ‡Infection with LCMV was performed at a multiplicity of infection (MOI) of three, 72 hours before the assay. On the day of the assay >95% of the cells were infected, as detected by immunofluorescence with MAb to LCMV nucleoprotein.

To establish the role of MHC glycoproteins, two approaches were used to modulate their levels: treatment with interferon-γ (IFN-γ) and transfection that led to expression of an exogenous MHC class I molecule, D^b. IFN-γ is a potent inducer of MHC expression in most cell types, including neurons (*9*). With the use of monoclonal antibodies (MAbs) to H-2^q molecules and flow cytofluorimetry, we found that OBL21 cells treated with recombinant IFN-γ were induced to express MHC class I molecules on their surfaces in a time- and concentration-dependent manner. Expression plateaued after 48 hours of treatment with 10 IU/ml. Interferon-treated LCMV-infected OBL21 cells, which expressed MHC class I molecules, were lysed by CTL (Table 1). Similarly, uninfected OBL21 cells were lysed when they received both IFN-γ and the appropriate H-2^q LCMV restricted chemically synthesized peptide (Table 1). Thus, the MHC glycoprotein is required for lysis and OBL21 cells are susceptible to CTL mediated injury.

When OBL21 cells were transfected to express D^b MHC class I molecules and then infected with LCMV, they were lysed by D^b-restricted, LCMV-specific CTLs, but not by H-2^q–restricted CTLs. However, treatment with IFN-γ restored lysis by H-2^q–restricted CTL and enhanced the killing by D^b-restricted CTL. The latter observation directly correlated with an enhanced expression of D^b molecules on the surfaces of these transfected cells (Table 2). The enhanced expression of the D^b was not caused by an increase of the mRNA from the transfection vector, as detected by Northern (RNA) blot analysis. Interferon-γ likely induced more efficient transport of D^b molecules to the cell surface. Peptide is required to stabilize the MHC class I heterodimer and allow their efficient transport to the cell's surface (*10*); a family of IFN-inducible genes may act as peptide transporters (*11*). This cell line may be down-regulated for these transporters as well as heavy chain mRNA.

The β_2-microglobulin (β_2M) subunit of the MHC class I molecule is necessary for MHC class I expression at the cell surface and for MHC function as a restriction element (*12*). Cotransfection with expression

Table 2. OBL21 neuronal cells expressing the D^b MHC class I molecule were lysed by D^b-restricted CTL in a CTL assay. OBL21 cells expressing functional D^b molecules were generated by calcium phosphate transfection with Mo-D^b (*19*). The plasmid pBSpac (*20*) was cotransfected to confer resistance to puromycin. Stable transfectants were selected with puromycin at 2 μg/ml. In the initial population, 20% of the cells expressed D^b on their cell surface by flow cytofluorimetry using MAb B22/249 (*18*). After three rounds of sorting, 80% of the cells stained positively for D^b. The data presented are the percent ^{51}Cr released from this cell population after a CTL assay as described in Table 1. When treated with 10 IU/ml of IFN-γ for 48 hours, the cells showed a mean fluorescence intensity of 420, baseline value was 36. Cells needed to be grown in puromycin and sorted frequently to maintain expression of the D^b. The CTL clone HL228 recognizes an epitope corresponding to aa 278 to 286 of the glycoprotein of LCMV. Similar results were obtained in three independent experiments.

Anti-LCMV CTLs	MHC restriction	E:T ratio	% ^{51}Cr released from OBL21 targets expressing D^b and treated with			
			None	LCMV	IFN-γ	IFN-γ LCMV
SWR/J day 7 Spl	q	50:1, 25:1	1, 0	7, 3	5, 1	36, 24
C57BL/6 day 7 Spl	b	50:1, 25:1	4, 1	20, 13	13, 6	54, 30
HL 228 CTL Clone	D^b	5:1, 2.5:1	5, 1	22, 13	3, 1	32, 15

vectors for the β_2M molecule (*13*) did not enhance the surface expression obtained with the D^b vector alone. When the endogenous mRNAs for heavy chain and for β_2M were measured by Northern blot analysis, heavy chain expression in OBL21 neuronal cells was 2% to 3% that of a control H-2^q fibroblast cell line (Fig. 2). In contrast, β_2M mRNA expression was equivalent in the OBL21 and fibroblast cell lines. Similarly, β_2M protein expression was equivalent in both OBL21 and fibroblast as detected by immunoprecipitation of OBL21 neuronal cells and H-2^q fibroblasts, after steady-state labeling with [^{35}S]methionine. The disassociation of MHC class I heavy chain transcription and translation from that of β_2M light chain was surprising, because both were previously found to be coregulated and share homologies in their promoter regions (*13*). IFN-γ treatment increased MHC heavy chain and β_2M mRNA in both OBL21 cells and H-2^q fibroblasts when compared to levels in untreated cells; in OBL21 cells the heavy chain increased to amounts normally found in untreated H-2^q fibroblasts.

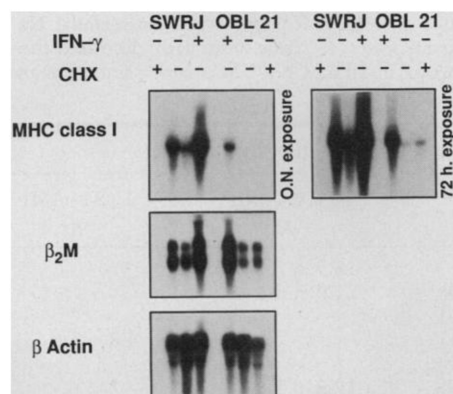

Fig. 2. A specific deficiency in expression of MHC class I heavy chain mRNA in OBL21 neuronal cells. A Northern blot containing RNA extracted from OBL21 neuronal cells and from SWR/J fibroblasts was probed for MHC class I heavy chain, β_2-microglobulin (β_2M), and β actin expression. Both cell types are H-2^q. Cell lines were untreated or treated with IFN-γ at 20 IU/ml for 48 hours or with cycloheximide (20 μg/ml) overnight. Total cytoplasmic RNA (10 μg) per lane was separated on a 1.5% agarose formaldehyde gel (*21*). RNA was transferred onto a Hybond-N membrane and hybridized successively with the three different probes. Labeling, hybridization, and stripping of the probes were done with standard protocols (*21*). The two upper panels represent different autoradiographic exposure times for the blot probed for MHC class I RNA (left, overnight; right, 72 hours). Probes for MHC class I heavy chains and for β_2M have been described (*22*). The probe for actin was a 300-bp fragment of mouse β actin cDNA.

OBL21 neuronal cells and H-2^q fibroblasts treated with cycloheximide (20 μg/ml) showed a four- to fivefold increase in mRNA for the heavy chain (Fig. 2), when compared to untreated cells, suggesting that this mRNA species was negatively regulated by a protein with a short half-life (*14*).

In summary, our studies indicate that neuronal cells do not express sufficient class I MHC molecules to serve as targets for CTL. The defect is in the amount of heavy chain expressed and can be overcome by transfection with an MHC class I heavy chain expression vector or induction with IFN-γ. This milieu provides a safe-house in which viruses can rest in neurons and avoid immunologic detection. The end result is a balance that may compromise neuronal function (*15*) but allow them to avoid destruction by killer T lymphocytes.

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