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A transgenic mouse model to assess the interaction of cytotoxic T lymphocytes with virally infected, class I MHC-expressing astrocytes

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Abstract

Astrocytes provide crucial support for neurons and their impairment by viruses or their interactions with anti-viral or autoimmune responses could contribute to neurological disease. We have developed a transgenic mouse model to assess lymphocyte–astrocyte interactions. The major histocompatibility complex (MHC) class I molecule, D^b, was expressed in astrocytes under the transcriptional control of regulatory sequences from the glial fibrillary acidic protein (GFAP) gene. Baseline cerebral MHC class I mRNA levels from transgenic mice were elevated over those of non-transgenic controls, and a prominent increase in cerebral MHC class I expression occurred following focal, injury-induced astroglial activation within transgenic brains but not in non-transgenic controls. FACS analysis of explant astrocyte cultures from established transgenic lines demonstrated astroglial expression of the GFAP-D^b fusion gene at the protein level. Functional antigen-presenting capacity was conferred by the D^b transgene, as virus-infected primary astrocytes obtained from transgenic BALB/c mice ($K^dI^dD^dL^d$) expressing the D^b molecule were lysed by D^b-restricted anti-viral CTL.

Key words: Astrocyte; Antigen presentation; CTL; Glial fibrillary acidic protein; LCMV; Major histocompatibility complex; Transgenic

1. Introduction

The central nervous system (CNS) has been considered an immune privileged site compared with other organs by virtue of the blood–brain barrier that restricts access of macromolecules and non-activated lymphoid cells from the periphery into the CNS parenchyma and because resident CNS cells such as neurons, oligodendrocytes and astrocytes express negligible to undetectable levels of class I major histocompatibility (MHC) molecules (reviewed in Lampson, 1987). MHC class I molecules function to present viral protein fragments (peptides) on the surface of cells so they can be recognized as foreign and be destroyed by MHC-matched, anti-viral CTL (Zinkernagel and Doherty, 1974; Townsend et al., 1985).

However, lack of MHC expression may not be absolute as astrocytes and oligodendrocytes can be focally induced to express MHC class I in response to certain CNS infections (Massa et al., 1986; Suzumura et al., 1986; Olsson et al., 1987; Liu et al., 1989) and during inflammatory demyelinating disease (Traugott and Lebon, 1988). Further, activated T lymphocytes can traverse the intact blood–brain barrier and enter the brain parenchyma (Wekerle et al., 1986, 1987; Hickey et al., 1991; Oldstone and Southern, 1992). Together, these data suggest that activated antiviral or autoimmune T cells can come in close proximity to resident CNS cells, and their interactions and/or release of cytokines may result in focal induction of MHC molecules on these CNS cells.

To better understand the potential role of class I MHC expression and antigen presentation by resident CNS cells, we have expressed a murine class I MHC molecule (D^b) in transgenic mice under the control of a variety of CNS cell-specific promoters. Here we report the establishment of transgenic mouse lines in which a constitutive and inducible class I MHC molecule is expressed in CNS astrocytes. Astrocytes provide crucial support for neurons and oligodendrocytes through such diverse functions as the production

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of neurotrophic factors and the elimination of neurotoxins (reviewed in Eddleston and Mucke, 1993). Consequently, their impairment or destruction by either antiviral or auto-immune responses would disturb CNS homeostasis. Notably, a variety of DNA and RNA viruses can infect astrocytes in vivo (Epstein et al., 1984; Stowring et al., 1985; Gyorkey et al., 1987; Mirra and del Rio, 1989; Carbone et al., 1991; Itoyama et al., 1991; Rinaman et al., 1993; Epstein et al., 1994). The GFAP-D^b mice should provide a useful tool to study immune interactions with virally infected astrocytes in vivo.

2. Materials and methods

2.1. Animals

Male and female C57Bl/6 and BALB/cByJ mice of various ages (newborns to 1 year of age) were used. Animals were maintained in conditions consistent with AAALAC regulations throughout the course of the investigation. Focal mechanical brain lesions were placed by penetration of one or both hemispheres with a sterile 27 gauge needle as described (Mucke et al., 1991). For this procedure, mice were anesthetized with methoxyflurane.

2.2. Cloning procedures and germline injection

Standard protocols (Ausubel et al., 1987; Sambrook et al., 1989) were used for the construction of the GFAP-D^b fusion gene. All junctions created by the subcloning were sequenced prior to microinjection. Fertilized oocytes were obtained from (C57Bl/6 × C57Bl/6) or (BALB/c × BALB/c) females. Purification of the transgene, preparation of mice and microinjection and reimplantation of fertilized oocytes were carried out as described (Mucke et al., 1991). The entire sequence of the GFAP gene was included in the construct to avoid deletion of potentially important intragenic regulatory elements (Sarkar and Cowan, 1991).

2.3. Northern analysis and molecular probes

Transgenic mice were identified by slot blot or Southern blot hybridization of genomic DNA extracted from tail tissue. Poly(A)$^+$ RNA was extracted from brains and analyzed by Northern blot and sequential hybridization with different probes (Mucke et al., 1991). The following DNAs were ^{32}P-labeled with the random hexanucleotide primer method (Sambrook et al., 1989) and used as probes: a partial D^b cDNA from clone pH 203 (Reyes et al., 1982) which cross-hybridizes with both H-2^b and H-2^d MHC class I transcripts (obtained from Dr. R.B. Wallace, City of Hope Research Institute, Duarte, CA); β2-microglobulin cDNA from clone p β2-m2 (Daniel et al., 1983), obtained from Dr. P. Kourilsky, Institute Pasteur, Paris, France; a cDNA fragment of mouse beta-actin bp 480–740 (Tokunaga et al., 1986) amplified from murine genomic DNA by PCR; and SV40 bp 1705–1910 (Lebowitz and Weissman, 1979) which identifies SV40 sequences at the 3′ end of the GFAP vector and serves as a marker for the transgene.

2.4. PCR detection of mRNA from tissues of transgenic and nontransgenic mice

Tissues were removed from transgenic and nontransgenic littermates and snap frozen in liquid nitrogen. Total RNA was later extracted using the GTC-acid phenol method (Chomczynski and Sacchi, 1987). 500 ng of total RNA was reverse transcribed (M-MLV reverse transcriptase, Gibco-BRL/Life Technologies, Gaithersburg, MD) at 37°C for 15 min using the random hexamer extension method (Perkin-Elmer Cetus, Emoryville, CA). The resulting cDNA was then subjected to 40 cycles of PCR (60°C annealing; 72°C extension; 95°C denaturation) in the presence of Taq polymerase and oligonucleotide primers designed to amplify either a product specific for the GFAP-D^b transgene (primers A and B) or an internal standard, GAPDH (primers C and D). The sequences of the primers are as follows: A, 5′ AGGTT GGAGC GGAGA CGCAT 3′; B, 5′ GCGCT CTGGT TGTAG TAGCC 3′; C, 5′ TGGTA TCGTG GAAGG ACTCA TGAC 3′; D, 5′ AGTCC AGTGA GCTTC CCGTT CAGC 3′.

2.5. Explant cultures of astrocytes

Primary astrocytes were isolated by a procedure modified from McCarthy and de Vellis (1980). Briefly, neonatal brains were mechanically dissociated in DMEM containing 15% fetal bovine serum. Following 3 days of incubation in poly-L-lysine coated tissue culture flasks, cells were shaken at 37°C at 100 rotations per min for 24 h to remove non-adherent cells. When fixed with 2% paraformaldehyde in PBS and labeled with antibodies against GFAP (DAKO, Carpinteria, CA) as described (Mucke et al., 1991), at least 95% of the adherent cells stained positive for this astroglial marker. All astrocytes were used within 15 days of culture and were passaged no more than three times.

2.6. FACS analysis

For FACS analysis, uninfected primary astrocytes were trypsinized, washed and incubated with medium alone or with a 1:50 dilution of primary antibodies:

B22.249 R1 (anti-D^b) or 30–5–7S (anti-L^d) (Accurate Chemical & Scientific Corporation, Westbury, NY). Binding of primary antibodies was revealed with a FITC-conjugated secondary antibody (dilution 1:200). All reactions were incubated on ice for 30 min. The cells were washed extensively and analyzed on a Becton Dickinson FACS 4. Dead cells were excluded by addition of 1 ng/ml propidium iodide to the samples prior to fluorimetry.

2.7. Chromium release assays

Chromium release assays were carried out as described elsewhere (Oldstone, 1990). In brief, primary astrocytes were infected with the Armstrong CA 1371 clone 53b of LCMV (LCMV Arm) (Dutko and Oldstone, 1983) at a multiplicity of infection (MOI) of 3. This MOI ensured that all cells became infected as determined by infectious center assays (not shown). 48 h later, the astrocytes were labeled with ^{51}Cr and exposed to LCMV primed H-2 matched or H-2 mismatched splenocytes at different effector-to-target cell (E:T) ratios. CTL specific for LCMV were raised by injecting 3–4-month-old C57Bl/6 and BALB/c mice intraperitoneally with 2×10^5 plaque-forming units of LCMV Arm. Splenic lymphocytes obtained from these mice 7–8 days later were used in chromium release assays. The specific ^{51}Cr release was calculated according to the following formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Each sample was done in triplicate and the variance within triplicates was 10% or less. Different groups of astrocytes compared in CTL assays were established and processed simultaneously.

3. Results

3.1. Establishment and identification of transgenic mice

To establish transgenic mice with astroglial expression of an MHC class I molecule, a minigene encoding the MHC class I antigen D^b was placed under the regulatory influence of a modified murine glial fibrillary acidic protein (GFAP) gene (Fig. 1). Earlier work documented that the GFAP gene effectively targets the expression of different proteins to astrocytes in vivo, including lacZ (Mucke et al., 1991), human alpha-1-antichymotrypsin (Mucke et al., 1992), the HIV1 coat protein, gp120 (Toggas et al., 1994), and the cytokine IL-6 (Campbell et al., 1993).

For construction of the GFAP-D^b transgene, the 5′ and 3′ ends of a partial cDNA encoding the greater portion of the D^b molecule (Reyes et al., 1982) were ligated with fragments of genomic D^b sequence derived from the Mo/D^b clone (Allen et al., 1986) (obtained from Dr. R. Flavell, Yale University, New Haven, CT). The resulting D^b minigene consists of all exons as well as the first two introns of the D^b gene. A polyadenylation signal is provided by 3′ untranslated genomic D^b sequence. The D^b minigene was fused with the modified GFAP gene (Mucke et al., 1991) after addition of *Not*I(5′) and *Sal*I(3′) linkers and the structure of the resulting construct was characterized by restriction analysis and sequencing of promoter-minigene junctions. The GFAP-D^b fusion gene was then microinjected into fertilized oocytes from C57Bl/6 (H-2^b) and BALB/c (H-2^d) mice as described (Mucke et al., 1991).

From the germline injections of the GFAP-D^b fusion gene into 200 fertilized oocytes, 11 transgenic founder mice were obtained. From five of these

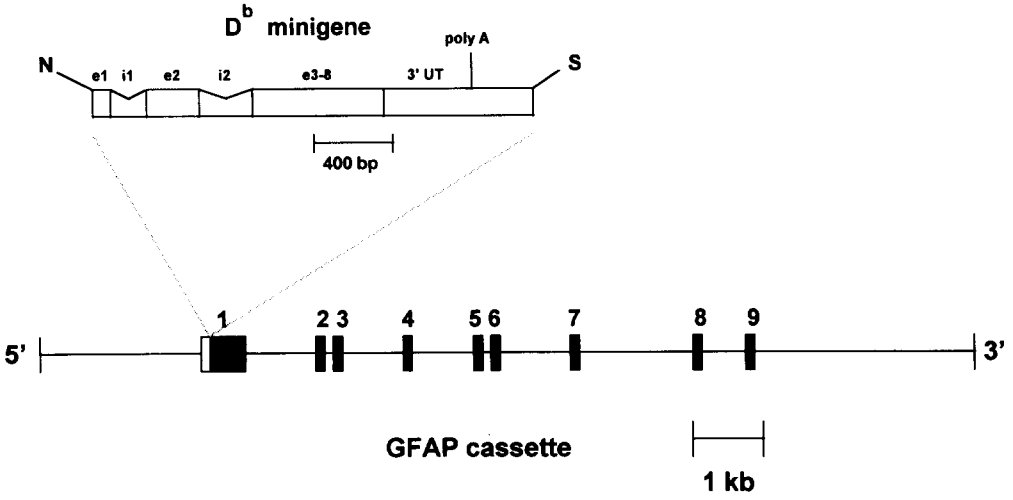

Fig. 1. Structure of the GFAP-D^b fusion gene. Numbers above black boxes identify exons of the GFAP gene. e, exon; i, intron; N, *Not*I; S, *Sal*I; UT, untranslated region.

founders, separate lines of transgenic mice were established and screened for expression of the transgene. Four of the five lines were derived from C57Bl/6 × C57Bl/6 (H-2^b) zygotes (lines nos. 162, 165, 184, 185) while one line (no. 208) was derived from a BALB/c × BALB/c (H-2^d) zygote.

3.2. mRNA and protein expression

To determine tissue expression of the GFAP-D^b transgene, reverse transcriptase/polymerase chain reaction (RT-PCR) was done on multiple tissues from transgenic and nontransgenic littermates including brain, spleen, pancreas, kidney, thymus, liver and testes. To determine RNA expression in the peripheral CNS, sciatic nerve was also analyzed. Two sets of primers were used. One set, primers A and B, shown in Fig. 2C, was specific for GFAP-D^b sequences and distinguished between spliced and unspliced products due to the presence of a 190-bp intron between the two primers. The other set of primers, primers C and D, served as a PCR control, and amplified GAPDH, a protein expressed in all tissues. Brain RNA from transgenic mice but not from nontransgenic littermates gave rise to a PCR product indicative of spliced RNA (Fig. 2A). A faint band was also detected in the thymi of these mice. No other organ, including sciatic nerve, gave rise to the transgene-derived PCR fragment. When the brain was dissected into four major regions (the cerebral hemispheres, the olfactory bulb, the cerebellum and the midbrain/brainstem), all portions of the brain resulted in the correctly spliced product (Fig. 2B). Thus, expression of the transgene is primarily restricted to the brain, and is expressed throughout the brain.

By Northern analysis, offspring from four (lines nos. 162, 165, 184, 208) of the five transgenic lines expressed GFAP-D^b at the RNA level (2–10 mice analyzed per line). At baseline, i.e. in unmanipulated animals, MHC class I mRNA levels were increased in

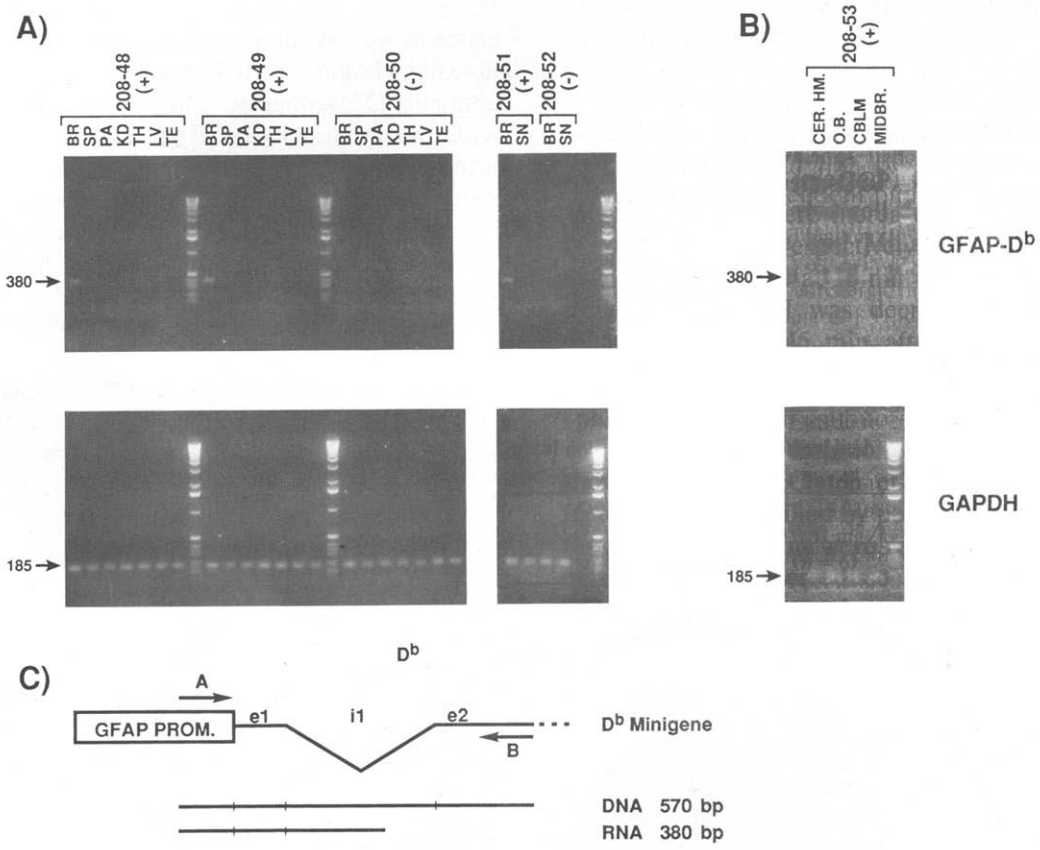

Fig. 2. Tissue distribution of mRNA from the GFAP-D^b transgene. Total RNA from tissues of transgenic (+) and nontransgenic (−) mice was extracted and subjected to RNA PCR following conversion to cDNA with reverse transcriptase as described in Materials and methods. Two sets of PCR primers were used: in the top panels, primers allowing amplification of the GFAP-D^b transgene were used; in the bottom panel, primers identifying an internal control, GAPDH, were used. **(A)** Tissue distribution of GFAP-D^b mRNA and of GAPDH in brain (BR), spleen (SP), pancreas (PA), kidney (KI), thymus (TH), liver (LV), testes (TE), and sciatic nerve (SN). The sizes of expected PCR products resulting from amplification are indicated: 380 bp for GFAP-D^b spliced RNA; 185 for GAPDH. **(B)** The brain of a transgenic mouse was dissected into the cerebral hemisphere (CER.HM.), the olfactory bulb (OB), the cerebellum (CBLM) and the midbrain (MDBR) and assessed by RT-PCR as described in Materials and methods. **(C)** Strategy of primer design to distinguish spliced mRNA from unspliced RNA or DNA PCR product. 'A' and 'B' refer to the primers used; e, exon; i, intron.

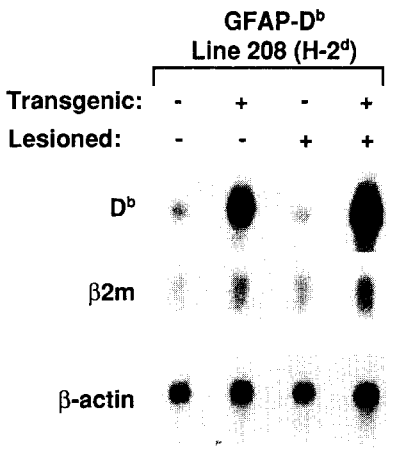

Fig. 3. Cerebral MHC class I mRNA levels in GFAP-D^b transgenic mice and non-transgenic controls. PolyA$^+$ RNA extracted from whole brains of GFAP-D^b transgenic mice (line 208) and controls (age- and strain-matched non-transgenic littermates) was immobilized on Northern blot and hybridized sequentially with different probes (indicated on left). Two mice received focal brain lesions, as described in Materials and methods, 2 days prior to sacrifice. Two unlesioned mice served to establish baseline levels of the different transcripts. A probe for beta-actin was used to assess amounts of RNA per lane.

the brains of GFAP-D^b transgenic mice compared with non-transgenic controls (Fig. 3). In normal non-transgenic mice, there was no detectable increase in the cerebral level of MHC class I mRNA 48 h following focal mechanical brain injury (Fig. 3). In GFAP-D^b transgenic mice, however, there was a marked increase in MHC class I mRNA levels in response to such injuries. This injury-induced upregulation of cerebral MHC class I expression was also found in transgenic lines nos. 162, 165 and 184 (2–6 mice analyzed per line) (data not shown).

The GFAP-D^b expression at the protein level was too low to be detected in situ by immunostaining. However, expression of the correctly folded D^b molecule on the surface of astrocytes could be demonstrated by FACS analysis (Fig. 4) using B22.249 R1, a monoclonal antibody that detects the correctly folded D^b molecule (Allen et al., 1986). An increase in mean fluorescence (from 12.26 to 23.24) was found in primary transgenic astrocytes relative to non-transgenic astrocytes (Fig. 4A, arrows). As a control, no significant difference in mean fluorescence was identified between transgenic and non-transgenic astrocytes when an L^d specific monoclonal antibody was used (Fig. 4B).

3.3. Transgene-derived D^b presents viral peptides to anti-viral CTL

The astroglial expression of a functional, transgene-derived D^b molecule was demonstrated by infection of explant cultures of astrocytes from transgenic mice with LCMV for 48 h followed by incubation of astrocytes with LCMV-specific, D^b-restricted CTL. Table 1 shows that astrocytes obtained from H-2^d BALB/c mice that expressed the H-2^b D^b minigene were specifically lysed by H-2^b CTL while astrocytes obtained from H-2^d mice not expressing the D^b molecule were not (36% vs. 10% ^{51}Cr release at E:T ratios of 50:1). Because the exposure to serum-containing media induces astrocytes to express endogenous MHC molecules (Keane et al., 1992), both transgenic and non-transgenic LCMV-infected astrocytes were effectively lysed by H-2-matched CTL bearing the same H-2 haplotype as the target cell (40% vs. 46% ^{51}Cr release at E:T ratios of 50:1). Similar results were obtained in

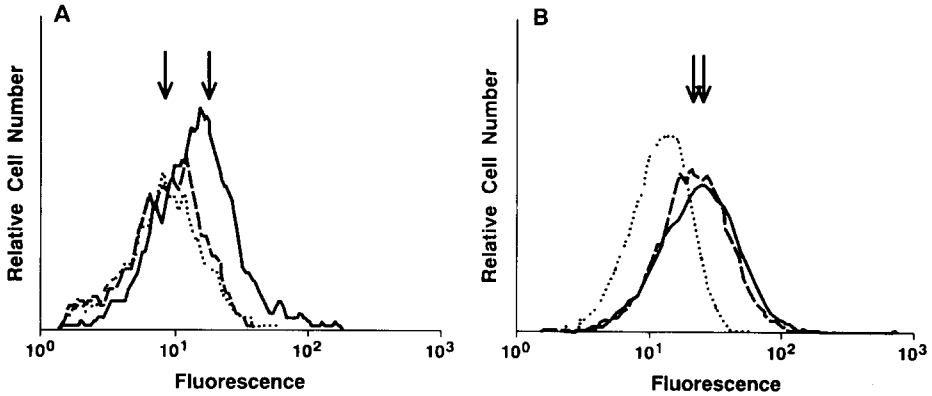

Fig. 4. Expression of MHC class I antigens on primary astrocytes from transgenic and non-transgenic mice. Primary astrocytes from transgenic (solid line) and non-transgenic (broken line) BALB/c (H-2^d) mice (line 208) were labeled with monoclonal antibodies directed against D^b (**A**) or L^d (**B**). Binding of primary antibodies was detected with fluorescein-conjugated secondary antibodies against mouse immunoglobulins. Cell surface fluorescence was determined on a flow cytometer as described in Materials and methods. In each panel, the dotted curve shows background staining of transgenic astrocytes incubated without primary antibody. Vertical arrows indicate mean fluorescence intensity of non-transgenic and transgenic astrocyte populations. Astrocytes were exposed to serum in culture media for 15 days prior to the FACS analysis which accounts for the expression of endogenous MHC molecules. FACS experiments were done in triplicate and results were equivalent in all instances.

Table 1
Chromium release assay of GFAP-D^b transgenic and non-transgenic BALB/c ($H\text{-}2^d$) astrocytes

Target	LCMV infection	$H\text{-}2^b$		$H\text{-}2^d$	
		50:1	25:1	50:1	25:1
Transgenic ($K^dI^dD^dL^d\text{-}D^b$)	–	13±3	8±2	5± 2	3± 2
Non-transgenic ($K^dI^dD^dL^d$)	–	11±4	5±3	3± 3	3± 1
Transgenic ($K^dI^dD^dL^d\text{-}D^b$)	+	36±8	29±5	40± 9	38± 9
Non-transgenic ($K^dI^dD^dL^d$)	+	10±2	8±1	46±11	35±10

Targets: GFAP-D^b transgenic and non-transgenic primary BALB/c ($H\text{-}2^d$) astrocytes, infected with LCMV Arm or mock infected 48 h prior to the CTL assay. Effectors: LCMV primed C57BL/6 ($H\text{-}2^b$) or BALB/c ($H\text{-}2^d$) splenocytes.
All samples analyzed in triplicate, using different astrocyte preparations. Values shown represent average % ^{51}Chromium released from labeled astrocytes with standard error indicated.

three other independent assays using three different batches of astrocytes.

4. Discussion

In this report, MHC class I minigene to astrocytes using sequences derived from the GFAP gene. Transgenic mice expressing D^b MHC molecules on astrocytes displayed similar survival rates as age and sex matched nontransgenic littermates over a 1-year period of observation (data not shown). Further, transgenic mice expressing the MHC molecule in astrocytes showed no signs of CNS impairment and histological analysis of their brains was indistinguishable from that of non-transgenic controls, suggesting that class I MHC expression alone is not sufficient to induce spontaneous neuropathology. The GFAP-D^b transgene is expressed in a functional manner as indicated by CTL assay (Table 1). LCMV-infected astrocytes from GFAP-D^b transgenic mice that are normally $K^dI^dD^d$ were specifically lysed by anti-viral, $H\text{-}2^b$ restricted CTL while astrocytes from normal BALB/c mice were not. Because the transgene-derived D^b molecule is the only $H\text{-}2^b$ MHC class I antigen that could be expressed on astrocytes of transgenic BALB/c ($H\text{-}2^d$) mice, lysis of these astrocytes by $H\text{-}2^b$-restricted CTL demonstrates that LCMV peptide processing and interaction with the D^b minigene product is appropriate for recognition by $H\text{-}2^b$ anti-LCMV CTL.

Transgenic mice which express a class I MHC gene product (K^b) driven by the GFAP promoter have been established previously (Schonrich et al., 1991). Our model incorporates the entire GFAP gene, which may include intragenic regulatory elements required for astrocyte-specific expression. Further, our choice of the D^b gene was chosen based on the well-characterized anti-LCMV response to target cells expressing the D^b molecule.

The inability to detect the D^b molecule in vivo with antibody likely reflects the insensitivity of the immunohistochemical assay since D^b was detected with antibody when FACS analysis was used in ex vivo astrocyte preparations (Fig. 4). Notably, CTL recognition and lysis despite undetectable levels of MHC class I and other immunoregulatory molecules have been noted (Skias et al., 1987; Oldstone et al., 1991).

A strength of this model is the inducibility of the GFAP promoter, exemplified here by the increase of transgenic class I MHC levels following mechanical trauma (Fig. 3). A variety of neural injuries have also been shown to up-modulate GFAP expression (Smith et al., 1983; de la Monte et al., 1987; Manuelidis et al., 1987; Delacourte, 1990).

Therefore, insults which result in an upregulation of the GFAP gene should correspond with focal induction of class I MHC expression on astrocytes. Experiments using allografts into the rat CNS and under the kidney capsule (Mason et al., 1986) have shown that rats mounted a brisk anti-graft response to the kidney graft while the CNS graft rejection occurred more slowly. This suggests that the microenvironment of the CNS, not the antigen-presenting capacity of the cells themselves, restricts immune recognition. Further, Massa has shown that brain-enriched gangliosides can suppress transcription of class I and II MHC on astrocytes (Massa, 1993). These results indicate that some cells of the CNS may be under immunosuppressive pressures that inhibit MHC expression resulting in a lack of recognition by the immune surveillance system and avoidance of immune-mediated damage. The ability to express inducible MHC molecules and cytokines in vivo in specific CNS cells by using CNS cell-specific promoters not sensitive to immunosuppressive factors should allow a determination of the immunopathogenic effects of aberrant MHC expression during diverse viral infections and autoimmune diseases of the CNS.

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