JOURNAL OF VIROLOGY, Nov. 1997, p. 8392–8396 Vol. 71, No. 11
0022-538X/97/$04.00+0

Virus-Specific, CD8$^+$ Major Histocompatibility Complex Class I-Restricted Cytotoxic T Lymphocytes in Lymphocytic Choriomeningitis Virus-Infected β_2-Microglobulin-Deficient Mice

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Received 7 May 1997/Accepted 19 August 1997

Following infection with lymphocytic choriomeningitis virus (LCMV), normal adult mice generate virus-specific, major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) which clear the virus after intraperitoneal infection or cause death following intracranial (i.c.) infection. We have investigated the response of β_2-microglobulin-deficient (β_2m^-) mice of the *H-2^d* haplotype (KOD mice) to LCMV infection. Unlike *H-2^b* β_2m^- mice, which generate CD4$^+$ MHC class II-restricted CTL in response to LCMV, KOD mice generate high levels of CD8$^+$ MHC class I-restricted, virus-specific CTL. These CTL are specific for the LCMV nucleoprotein epitope (residues 118 to 126) in association with the L^d class I molecule, analogous to the CTL response in wild-type mice. KOD mice are also susceptible to lethal LCM disease, with 75 to 80% of the mice dying 7 to 9 days following i.c. infection with virus. Similar to results with normal mice, lethal LCM disease in KOD mice is prevented by in vivo depletion of CD8$^+$ T cells prior to i.c. infection. In contrast to wild-type mice, however, KOD mice cannot control LCMV and become persistently infected. Overall, these results demonstrate that β_2m is not an absolute requirement for presentation of endogenous antigen on L^d or for induction of virus-specific L^d-restricted CTL in vivo.

Major histocompatibility complex (MHC) class I molecules bind peptides of generally 8 to 10 amino acids in length which are derived from endogenously synthesized proteins (28, 33). These peptides are translocated from the cytosol into the endoplasmic reticulum via the heterodimeric transporter associated with antigen processing (TAP). In the endoplasmic reticulum, antigenic peptide binds to the MHC class I heavy-chain and the noncovalently associated light-chain β_2-microglobulin (β_2m) (15). The resulting trimolecular complex transits to the cell surface via the secretory pathway, where it can be recognized by CD8$^+$ cytotoxic T lymphocytes (CTL).

Experiments using β_2m-deficient (β_2m^-) mice (17, 38) have conclusively demonstrated the crucial role of β_2m in antigen presentation to CD8$^+$ T cells. β_2m^- mice are deficient in cell surface expression of correctly conformed MHC class I molecules, and as a consequence, they are defective in thymic selection of CD8$^+$ T cells. These mice, therefore, have negligible numbers of peripheral CD8$^+$ cells (17, 38), and unlike normal mice, β_2m^- mice typically fail to generate MHC class I-restricted CTL in response to virus infection. Instead, β_2m^- mice generate CD4$^+$ MHC class II-restricted CTL following infection with Sendai virus (16) or with lymphocytic choriomeningitis virus (LCMV) (11, 20, 23, 27). In contrast to what occurs after viral infection, injection of β_2m^- mice with allogeneic β_2m^+ cells results in the expansion of highly cytolytic CD8$^+$ T cells (3, 4, 18, 26, 31). This demonstrates that, if given a sufficiently potent stimulus, β_2m^- mice can elaborate functional CD8$^+$ CTL. It has also been shown that β_2m^- cells express low levels of correctly conformed MHC class I molecules on their surfaces (1, 6) and furthermore that the class I molecules expressed on β_2m^- cells can present endogenous antigen to CD8$^+$ T cells from normal mice (14, 19, 26, 39). These findings suggest that the presentation of certain endogenously derived antigens on "free" (non-β_2m-associated) MHC class I heavy chains might result in induction of CD8$^+$ CTL in β_2m^- mice. To address this possibility, we have investigated the antiviral CTL responses of different strains of β_2m^- mice. In this report we demonstrate the induction of high levels of CD8$^+$, MHC class I-restricted CTL activity following LCMV infection of *H-2^d* β_2m^- (KOD) mice.

MATERIALS AND METHODS

Virus. The Armstrong-3 strain of LCMV was originally obtained from J. L. Whitton (The Scripps Research Institute, La Jolla, Calif.) and was propagated in BHK-21 cells (American Type Culture Collection, Rockville, Md.). Infectious supernatants were titrated on Vero cell monolayers as previously described (37).

Mice. β_2m^- mice were crossed with BALB/c mice to generate *H-2^d* β_2m^- mice (KOD mice). These mice were obtained from F. Wells (National Cancer Institute, Frederick, Md.) (34) and were maintained in specific-pathogen-free, AAALAC-accredited facilities at the University of North Carolina, Chapel Hill, and at Loyola University Medical Center, Maywood, Ill. C57BL/6J (B6; *H-2^b*), C57BL/6J-B2m^{tm1UNC} (B6.β_2m^-), AKR/J (*H-2^k*), AKR/J-B2m^{tm1UNC} (AKR.β_2m^-), and BALB/cJ mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Male and female mice were used at 7 to 14 weeks of age. Mice were infected via the intraperitoneal (i.p.) route with either 2×10^2 or 2×10^4 PFU of LCMV in a volume of 0.4 ml. Spleens were removed from mice either 20 days or 120 days following i.p. infection with LCMV for determination of virus titers. The spleens were homogenized, and virus titers in the supernatants were measured by plaque assay on Vero cell monolayers as previously described (37). For intracranial (i.c.) infections, mice were anesthetized with Metofane (Pitman-Moore Inc., Mundelein, Ill.) and injected i.c. with either 2×10^3 or 2×10^5 PFU of LCMV in a volume of 20 μl. Following i.c. infection, mice were monitored daily for morbidity and mortality.

Cell lines. C1R cells transfected with *H-2L^d* (C1R-L^d) and the control cell line C1R-neo were obtained from P. Cresswell (Yale University School of Medicine, New Haven, Conn.) (10) and were maintained in D-10 (Dulbecco's modified Eagle's medium containing 10% fetal calf serum [FCS] and 50 μM 2-mercapto-

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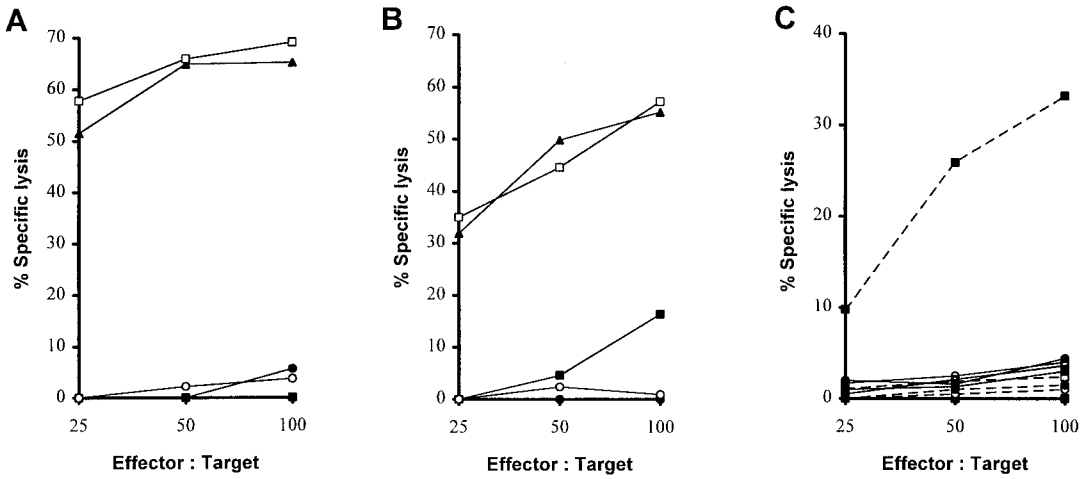

FIG. 1. KOD mice generate LCMV NP-specific, L^d-restricted CTL in response to virus infection. BALB/cJ mice (A) and KOD mice (B) were infected i.p. with 2×10^4 PFU of LCMV. Eight days later, spleen cells from these mice were tested for cytotoxic activity against a panel of cell lines by standard ^{51}Cr-release assays. The cell lines tested were C1R-neo (●), C1R-L^d/p8901 (■), C1R-neo/NP (○), and C1R-L^d/NP (□) and C1R-L^d (▲) which was incubated with the NP residue 118 to 126 peptide prior to assay. Similar results were obtained following i.p. infection of mice with 2×10^2 PFU of LCMV. (C) AKR/J mice (broken lines) and AKR.β_2m^- mice (solid lines) were infected i.p. with 2×10^4 PFU of LCMV. Eight days following infection, spleen cells from these mice were tested for cytotoxic activity against L cells (*H-2*^{k}, ■ and □) or MC57 cells (*H-2*^{b}, ● and ○) that were either infected with LCMV (■ and ●) or mock infected (□ and ○). Representative results from four (A and B) and three (C) independent experiments are shown.

ethanol), supplemented with 1 mg of G418 (Gibco BRL, Grand Island, N.Y.) per ml. The C1R-L^d/NP and the C1R-neo/NP cell lines were generated as follows. The pARM NP-A construct containing the LCMV Armstrong-3 nucleoprotein (NP) gene was obtained from J. L. Whitton (35). The *Not*I-*Sph*I restriction fragment encoding NP was excised from pARM NP-A and was inserted into the *Bam*HI site of the episomal vector p8901 (2) by blunt-end ligation. The resulting p8901-NP construct was transfected into C1R-L^d and C1R-neo cells by electroporation with a Bio-Rad Gene Pulser. Transfected cells were selected with D-10 supplemented with 1 mg of G418 per ml and 2 mg of hygromycin B (Calbiochem, San Diego, Calif.) per ml. NP expression in these C1R-L^d/NP and C1R-neo/NP transfectants was confirmed by immunocytochemistry with an NP-specific monoclonal antibody generously provided by M. J. Buchmeier (25). The C1R-L^d cell line was also transfected with the p8901 vector as a control (C1R-L^d/p8901).

The fibroblastoid MC57 (*H-2*^{b}) and L cell (*H-2*^{k}) lines have been previously described (23). These cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 50 μM 2-mercaptoethanol.

Peptide synthesis. The immunodominant LCMV epitope recognized by CTL from *H-2*^{d} mice has been mapped to residues 118 to 126 of the viral NP (30, 36). The peptide sequence corresponding to this region (RPQASGVYM) was synthesized by the University of North Carolina Peptide Synthesis Facility with a Bioresearch 9500 automated synthesizer and was purified by high-performance liquid chromatography before use.

Cytotoxicity assays. Spleens were removed from mice 8 days following i.p. infection with LCMV. The spleens were disrupted and erythrocytes were removed by osmotic lysis. These effector cells were assayed for LCMV-specific CTL activity by standard ^{51}Cr-release assays as previously described (37). The target cell lines used in these assays were the C1R-neo/p8901, C1R-L^d/p8901, and C1R-L^d/NP cell lines described above, as well as LCMV-infected and mock-infected MC57 and L cell lines. Infection of MC57 cells and L cells with LCMV was performed exactly as described previously (23, 26). In some experiments C1R-neo/p8901 and C1R-L^d/p8901 cells were incubated with 2 μM NP residue 118 to 126 peptide at 37°C for 1 h after being loaded with ^{51}Cr. Unbound peptide was removed by washing the cells with medium containing 5% FCS, and these peptide-pulsed cells were then used as targets in ^{51}Cr-release assays. Effector cells were incubated with the target cells for 5 h at 37°C in 5% CO_2. Supernatants were harvested with a Skatron Instruments (Sterling, Va.) harvesting system, and the amount of ^{51}Cr released was quantified with a γ-counter (Packard, Sterling, Va.). All assays were performed in triplicate, and the percent specific lysis was determined by the formula percent specific lysis = [(cpm released in the presence of effectors − spontaneous release)/(total release − spontaneous release)] × 100, where total release is the counts per minute (cpm) released from target cells by addition of 5% (vol/vol) Triton X-100 (final concentration) and spontaneous release is the counts per minute released from target cells in the absence of effectors.

Depletion of T-cell subsets. In vitro T-cell-depletion experiments were performed as previously described (37) except that the antibodies used in this study were 174.2 (anti-murine CD4) (7) and 31M-6 (anti-murine CD8) (29). LCMV-specific CTL activity of effector cells depleted of specific T-cell subsets was measured by standard ^{51}Cr-release assays as described above. Depletion of T cells in vivo was done with the antibodies GK1.5 (CD4) and 53-6.72 (CD8) as previously described (27).

Flow cytometry. Effector cells were prepared from the spleens of mice 8 days following i.p. infection with 2×10^4 PFU of LCMV as described above. Cells were resuspended at 10^7 cells/ml in RPMI 1640 containing 5% FCS. Aliquots of the cell suspension (5×10^5 cells) were taken and incubated with the monoclonal antibody 2.4G2 (32) at a concentration of 0.4 μg/10^6 cells to block Fc receptors. The cells were then stained with phycoerythrin-conjugated anti-CD4 (clone RM4-5; Pharmingen, San Diego, Calif.) and fluorescein isothiocyanate-conjugated anti-CD8 (clone 53-6.7; Pharmingen) at a concentration of 1 μg/10^6 cells for 25 min at 4°C. After being washed three times with ice-cold phosphate-buffered saline containing 1% (vol/vol) FCS and 15 mM sodium azide, the cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). Dead cells were excluded on the basis of forward and side scatter, and data analysis was performed on 20,000 acquired events with the computer program Cicero (Cytomation, Fort Collins, Colo.).

RESULTS AND DISCUSSION

The CTL response of BALB/cJ mice to LCMV is dominated by L^d-restricted, $CD8^+$ CTL (24). Furthermore, it has been estimated that >95% of the CTL induced by LCMV infection of BALB/c mice recognize the NP residue 118 to 126 peptide bound to L^d (36). We examined whether KOD mice generated L^d-restricted, NP-specific CTL following infection with LCMV. As expected, spleen cells taken from BALB/cJ mice 8 days after i.p. infection with either 2×10^2 or 2×10^4 PFU of LCMV had high levels of CTL activity against C1R-L^d/NP cells (Fig. 1A). Surprisingly, we found that spleen cells from LCMV-infected KOD mice, like spleen cells from BALB/cJ mice, also killed C1R-L^d/NP cells (Fig. 1B). The control cell lines C1R-L^d/p8901 and C1R-neo/NP were not killed by these CTL, indicating that the LCMV-induced CTL activity in KOD mice is both antigen specific and MHC restricted. We obtained similar results using LCMV-infected and noninfected L cells (*H-2*^{k}) transfected with L^d as target cells (data not shown). Like the BALB/cJ-derived CTL, the LCMV-specific CTL from KOD mice recognized the immunodominant L^d-restricted NP 118 to 126 epitope. C1R-L^d cells that had been incubated with NP 118 to 126 were killed efficiently by spleen cells from LCMV-infected BALB/cJ mice (Fig. 1A) and by spleen cells from LCMV-infected KOD mice (Fig. 1B). Thus, following

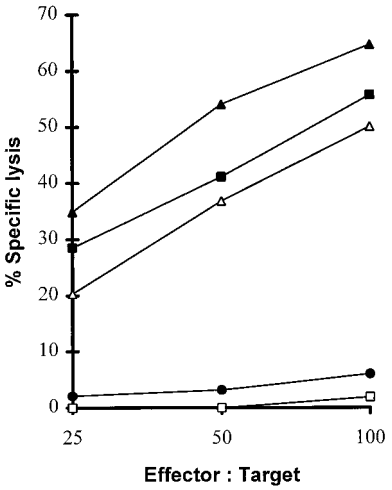

FIG. 2. LCMV-specific L^d-restricted CTL in KOD mice are $CD4^-$ $CD8^+$. Spleen cells were prepared from LCMV-infected KOD mice 8 days after i.p. infection with 2×10^4 PFU of LCMV. Prior to use in CTL assays, spleen cells were treated with complement only (■), anti-CD4 plus complement (△), or anti-CD8 plus complement (□) or were left untreated (▲ and ●). Target cells were C1R-L^d/NP (▲, ■, △, and □) or C1R-neo/NP (●). The LCMV-specific CTL activity in the spleens of LCMV-infected KOD mice is completely removed by treatment with anti-CD8 plus complement. Depletion of $CD4^+$ cells has little effect on the level of CTL activity. Representative results from one of three independent experiments are shown.

LCMV infection, KOD mice generated L^d-restricted, NP-specific CTL. We have previously reported that *H-2*^{b} β_2m^- mice do not generate MHC class I-restricted CTL following LCMV infection (23). Furthermore, unlike AKR/J mice, AKR.β_2m^- mice (*H-2*^{k}) do not generate MHC class I-restricted CTL following i.p. infection with LCMV (Fig. 1C). Thus, the ability of β_2m^- mice to generate LCMV-specific, MHC class I-restricted CTL following virus infection seems to be restricted to β_2m^- mice of the *H-2*^{d} haplotype.

We performed in vitro complement depletion experiments to determine the phenotype of the KOD CTL responsible for the virus-specific, L^d-restricted cytotoxic activity. Treatment of spleen cells from LCMV-infected KOD mice with antibodies against CD4, followed by complement, had no effect on the LCMV-specific, L^d-restricted CTL activity. In contrast, treatment of these spleen cells with anti-CD8 followed by complement totally removed the LCMV-specific, L^d-restricted cytotoxic activity (Fig. 2). These data indicate that the virus-specific, L^d-restricted CTL response following LCMV infection of KOD mice like the response of wild-type mice, is mediated by $CD4^-$ $CD8^+$ T cells. We next examined the spleens of KOD mice for evidence of expansion of $CD8^+$ T cells in response to LCMV infection. Eight days after i.p. infection of control BALB/cJ mice with 2×10^4 PFU of LCMV, there was an approximately twofold increase in the percentage of splenic $CD8^+$ cells (Fig. 3A and B). Less than 1% of the spleen cells from noninfected KOD mice expressed CD8 (Fig. 3C); however, 8 days after LCMV infection of these mice there was almost a fivefold increase in the percentage of splenic $CD8^+$ cells (Fig. 3D). Taken together with the CTL assay results, these data indicate that LCMV infection of KOD mice results in expansion of virus-specific, $CD8^+$, L^d-restricted CTL.

The CTL response of KOD mice to LCMV differs from that observed in *H-2*^{b} β_2m^- mice. We have previously reported that *H-2*^{b} β_2m^- mice generate $CD4^+$ MHC class II-restricted CTL in response to LCMV infection (23, 27, 37). We were unable to detect LCMV-specific, MHC class I-restricted T cells in these mice even following in vitro restimulation of spleen cells or by testing more than 300 T-cell hybridomas generated with splenocytes from B6.β_2m^- mice (*H-2*^{b}) which had been acutely infected with LCMV (data not shown). Furthermore, an increase in splenic $CD8^+$ cells in B6.β_2m^- mice following LCMV infection was not detected (36a). These data are con-

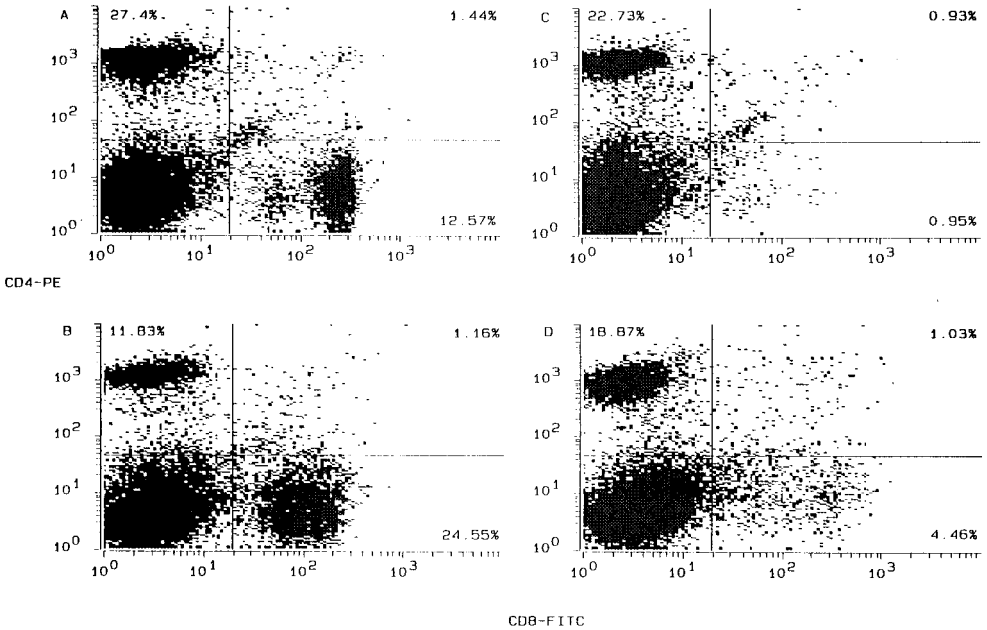

FIG. 3. Expansion of $CD8^+$ cells following LCMV infection of KOD mice. BALB/cJ (A and B) and KOD (C and D) mice were either injected i.p. with 2×10^4 PFU of LCMV (B and D) or left untreated (A and C). Eight days later, spleen cells were prepared from these mice and were stained with phycoerythrin (PE)-conjugated anti-CD4 and with fluorescein isothiocyanate (FITC)-conjugated anti-CD8. Compared with results with the noninfected mice, there was an expansion of $CD4^-$ $CD8^+$ cells in both strains of mice following LCMV infection. Representative results from one of three independent experiments are shown.

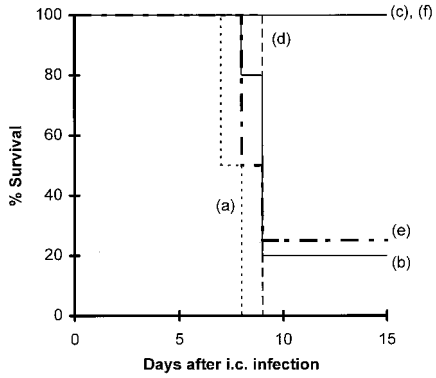

FIG. 4. Lethal LCM disease in KOD mice is dependent on $CD8^+$ cells. BALB/cJ (a) ($n = 4$) and KOD (b to f) mice were injected i.c. with either 2×10^3 PFU (a to d) or 2×10^5 PFU (e) of LCMV or with an equivalent volume of phosphate-buffered saline (f) ($n = 5$). Prior to infection, selected groups of KOD mice were depleted of $CD8^+$ (c) ($n = 8$) or $CD4^+$ (d) ($n = 5$) cells by injection of specific antibody (27). Mice were monitored daily for morbidity and mortality. In the experiment whose results are shown, 8 of 10 (b) and 6 of 8 (e) nondepleted, KOD mice died following i.c. infection with 2×10^3 and 2×10^5 PFU of LCMV, respectively.

sistent with results of a previous study showing that spleens from noninfected as well as from LCMV-infected *H-2*^{b} β_2m^- mice contain less than 1% $CD8^+$ cells (8). Therefore, although *H-2*^{b} β_2m^- cells express MHC class I molecules, particularly D^b (1, 6), which can present endogenously derived antigen to $CD8^+$ T cells from normal mice (14, 19, 26, 39), the level of expression of MHC class I complexes in B6.β_2m^- mice is not sufficient to elicit a $CD8^+$ T-cell response in vivo. Similarly, we were also unable to detect LCMV-specific, MHC class I-restricted CTL in AKR.β_2m^- mice (Fig. 1C). Thus, the L^d class I molecule appears unique in its ability to function efficiently in the absence of β_2m. Our results are in agreement with the findings of Cook et al. (9). Using a different strain of KOD mice, those authors demonstrated the induction of antigen-specific $CD8^+$ CTL following injection of L^d-binding peptides. This result indicates that the level of L^d expression on the surfaces of β_2m^- cells in vivo is sufficient to elicit a MHC class I-restricted CTL response. Our data from infection with virus rather than injection of peptides extend these observations. The induction of virus-specific, L^d-restricted $CD8^+$ CTL following LCMV infection of KOD mice indicates that, in the absence of β_2m, the level of expression of endogenously derived NP residues 118 to 126 in association with L^d is sufficient to induce a CTL response in vivo. These data also indicate that, at least for NP residues 118 to 126, β_2m is not an absolute requirement for the presentation of endogenous antigen in association with L^d.

We next examined whether the virus-specific $CD8^+$ CTL in KOD mice are functional in vivo. Following i.c. infection with LCMV, normal mice succumb to aseptic meningitis and die 6 to 9 days following infection. This lethal disease is mediated by LCMV-specific $CD8^+$ MHC class I-restricted CTL (21, 22). i.c. infection of KOD mice with either 2×10^3 or 2×10^5 PFU of LCMV resulted in death of 8 of 10 and 6 of 8 animals, respectively, 7 to 9 days after i.c. infection (Fig. 4). The symptoms of LCM disease in KOD mice were similar to those observed in i.c. infected BALB/cJ mice. In vivo depletion of $CD8^+$ cells prior to i.c. infection prevented lethal LCM disease in KOD mice, whereas depletion of $CD4^+$ cells had no effect on disease (Fig. 4). Thus, the disease observed following i.c. infection of KOD mice with LCMV is similar to that seen in i.c. infected wild-type mice. Furthermore, the observation that KOD mice

TABLE 1. KOD mice do not clear LCMV

Mouse strain	Days after infection[a]	Log_{10} PFU/g of tissue[b]	
		Expt 1	Expt 2
KOD	Noninfected	<3	<3
BALB/cJ	20	<3	<3
	120	<3	<3
KOD	20	6.90	7.10
	120	7.00	6.95
AKR.β_2m^-	120	7.16	7.23
B6.β_2m^-	120	7.23	7.00

[a] Mice were infected i.p. with 2×10^4 PFU of LCMV in a volume of 0.4 ml.
[b] Values are the levels of virus detected in the spleens of individual mice. The limit of detection of the assay is 3 log_{10} PFU/g.

succumb to $CD8^+$ T-cell-dependent lethal LCM disease suggests that the virus-specific $CD8^+$ CTL from these mice that we detected in vitro are functional in vivo. Since clearance of LCMV is also dependent on $CD8^+$ CTL (20, 37), we examined whether KOD mice could control LCMV infection. As shown in Table 1, KOD mice which were infected i.p. with 2×10^4 PFU of LCMV contained high levels of virus in their spleens 20 days and 120 days after infection. Moreover, by day 20 following i.p. infection with 2×10^2 PFU of LCMV, the virus had disseminated and was detectable in the spleen, liver, and brain (Table 2). Interestingly, none of the strains of β_2m^- mice that we examined were capable of clearing LCMV infection (Table 1). This result is not surprising for B6.β_2m^- mice and AKR.β_2m^- mice, which lack detectable MHC class I-restricted CTL activity following LCMV infection. In contrast, infection of KOD mice with 2×10^2 or 2×10^4 PFU of LCMV resulted in high levels of virus-specific, MHC class I-restricted CTL activity, yet these mice also failed to eliminate LCMV. The inability of the virus-specific L^d-restricted CTL in KOD mice to clear LCMV infection is puzzling. The failure of KOD mice to clear LCMV contrasts with the antiviral response observed in BALB/c-*H-2*^{dm2} mice. BALB/c-*H-2*^{dm2} mice lack L^d and generate low levels of LCMV-specific CTL activity following infection. Unlike KOD mice, BALB/c-*H-2*^{dm2} mice clear LCMV infection (13). In addition, the low levels of virus-specific, MHC class I-restricted CTL detectable in CD8-deficient mice (5) are also sufficient to clear LCMV infection (12). Because MHC class I-restricted CTL activity is readily detectable in LCMV-infected KOD mice, unlike in BALB/c-*H-2*^{dm2} and CD8-deficient mice, it is unlikely that the inability of KOD mice to control LCMV infection is due to insufficient numbers of MHC class I-restricted CTL. An alternative possibility is that in KOD mice, LCMV persists in cells that do not express sufficient levels of L^d to be recognized and killed by virus-

TABLE 2. Dissemination of virus in LCMV-infected KOD mice

Mouse strain	Days after infection[a]	Log_{10} PFU/g of tissue in[b]:		
		Spleen	Liver	Brain
KOD	Noninfected	<3	<3	<3
BALB/cJ	20	<3	<3	<3
	20	<3	<3	<3
KOD	20	7.0	6.6	4.4
	20	7.2	7.2	6.1
	20	7.8	8.0	5.6

[a] Mice were infected i.p. with 2×10^2 PFU of LCMV in a volume of 0.4 ml.
[b] Values are the levels of virus detected in tissues of individual mice 20 days following i.p. infection with LCMV. The limit of detection of the assay is 3 log_{10} PFU/g.

specific CTL. This possibility is consistent with the observation that the level of expression of MHC class I on β_2m^- fibroblasts is substantially lower than that on β_2m^- T cells (25a). LCMV-infected cells that express insufficient levels of L^d for CTL recognition may, therefore, act as a reservoir of virus in vivo.

In conclusion, we have demonstrated that KOD mice generate NP-specific, $CD8^+$, L^d-restricted CTL following virus infection. This is the first report of MHC class I-restricted, $CD8^+$ CTL induction in β_2m^- mice in response to virus infection. The ability of KOD mice to generate $CD8^+$, L^d-restricted CTL indicates that β_2m expression is not an absolute requirement for the presentation of endogenous peptide antigen in association with L^d.

ACKNOWLEDGMENTS

D.G.Q. and A.J.Z. contributed equally to this work.

We thank Kathie Lindley for maintenance of the mice.

This work was supported in part by the Stritch School of Medicine, Loyola University (D.G.Q.) and by NIH grant AI 20288 (J.A.F.). A.J.Z. was a recipient of a graduate fellowship from the Lineberger Comprehensive Cancer Center.

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