Theiler’s Virus and Mengo Virus Induce Cross-Reactive Cytotoxic T Lymphocytes Restricted to the Same Immunodominant VP2 Epitope in C57BL/6 Mice

SVEN DETHLEFS,1 NICOLAS ESCRIOU,2† MICHEL BRAHIC,1 SYLVIE VAN DER WERF,2 AND EVA-LOTTA LARSSON-SCIARD1†

Unite´ des Virus Lents, URA 1157 Centre National de la Recherche Scientifique,1 and Unite´ de Genetique Mol´eculaire des Virus Respiratoires,2 Institut Pasteur, 75724 Paris Cedex 15, France

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C57BL/6 mice develop a virus-specific cytotoxic T-lymphocyte (CTL) response after intraperitoneal inoculation with either the DA strain of Theiler’s virus or Mengo virus, two members of the Cardiovirus genus. These CTLs contribute to viral clearance in the case of Theiler’s virus but do not protect the mice from the fatal encephalitis caused by Mengo virus. In this study we show that DA and Mengo virus-induced CTLs are cross-reactive. The cross-reactivity is due to a conserved, H-2Dβ-restricted epitope located between amino acid residues 122 and 130 of the VP2 capsid protein (VP2122-130). This epitope is immunodominant in C57BL/6 mice infected with Theiler’s virus. The VP2122-130 epitope, initially identified for Mengo virus, is the first CTL epitope described for Theiler’s virus.

The Cardiovirus genus within the family of Picomaviridae contains two serologically distinct subgroups: Theiler’s murine encephalomyelitis virus (TMEV) and the encephalomyocarditis virus (EMCV) group, which includes Mengo virus. The DA strain of TMEV (DA virus) is a natural murine pathogen which causes a persistent infection of the central nervous system (CNS) in susceptible mice (18). This chronic CNS disease is characterized by infection of macrophage-microglial cells, oligodendrocytes, and astrocytes and the development of demyelination (1, 9, 19). The histopathological findings parallel those of multiple sclerosis in humans and therefore make TMEV infection an animal model for this CNS disease. The H-2Dβ region of the major histocompatibility complex (MHC) plays a key role (8, 26, 28) in the susceptibility to persistent TMEV infection. The H-2Dβ haplotype is associated with resistance, whereas the H-2k, H-2q, and H-2b haplotypes are associated with susceptibility (6). The gamma interferon locus on chromosome 10 and a gene close to the Mbp locus on chromosome 18 are also involved (5). The major contribution of the MHC D region indicates that class I-restricted T cells may play a crucial role in the host’s defense against TMEV. This was supported by experiments which showed that susceptible H-2k FVB mice transgenic for the H-2Dβ gene clear TMEV infection and are protected against the demyelinating disease (2). Also, H-2b mice that lack the β₂-microglobulin gene and do not express stable MHC class I molecules are susceptible to persistent TMEV infection (13, 23, 27). In a recent study, Lin et al. showed that both the VP1 and VP2 capsid proteins are targets for TMEV-restricted cytotoxic T lymphocytes (CTLs) in B10 (H-2b) mice, but the epitopes were not determined (17). The M strain of Mengo virus belongs to the EMCV group of the Cardiovirus genus. It is highly neurotropic and causes a fatal encephalitis (10). Attenuated strains of Mengo virus (vMC24 and vMC0) have been generated by the partial or total deletion of the poly(C) tract of the 5′ noncoding region of the viral genome (11, 22). Mice vaccinated with vMC24 develop a strong virus-specific humoral and MHC class I-restricted CTL response and are protected against a subsequent lethal challenge with the wild-type Mengo virus (11). We have previously demonstrated that vMC24-induced CDS1 T lymphocytes are H-2Dβ restricted in C57BL/6 mice (12). Mapping experiments using recombinant vaccinia viruses and peptide scanning revealed that these CTLs are restricted to the C-terminal part of the VP2 capsid protein (12) and that two epitopes, the VP epitope between amino acid residues 122 and 130 (VP2122-130) and the VP2146-154 epitope, are immunodominant (12a). The VP2122-130 peptide is conserved between Mengo virus and TMEV (Fig. 1).

In this study we demonstrate that CTLs induced by TMEV and Mengo virus in C57BL/6 mice are cross-reactive and that this cross-reactivity can be mapped to the VP2122-130 epitope. We show that this epitope is immunodominant for the induction of TMEV-specific CTLs. Our findings constitute the first identification of an immunodominant CTL epitope for TMEV infection in a resistant mouse strain.

MATERIALS AND METHODS

Animals and cell lines. C57BL/6 female mice (6 to 8 weeks old) were purchased from Iffa Credo or Janvier and kept in the animal facilities of the Institut Pasteur, Paris, France. KSSV (H-2b) and C57SV (H-2b) fibroblasts were a gift from B. Knowles, Jackson Laboratory, Bar Harbor, Maine. Transfectant Ltk− cell lines expressing either the H-2Kb or the H-2Dβ MHC class I molecules were kindly provided by P. Langlade-Demoyen, Institut Pasteur. The cells were maintained in RPMI 1640 supplemented with 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, antibiotics, and 5% heat-inactivated fetal calf serum (RPMI 1640 culture medium).

Generation of CTLs. Mice were inoculated intraperitoneally with 10⁶ PFU of DA virus or with 10⁶ PFU of attenuated Mengo virus (strain VMC0). Three weeks after inoculation, spleen cells were harvested and seeded into upright T75 flasks (Costar) at 2 × 10⁶ cells/ml in RPMI 1640 culture medium containing 5 × 10⁻⁵ M 2-mercaptoethanol. The cells were restimulated in vitro by adding either DA virus-infected (>5 PFU/cell) or Mengo virus-infected (>5 PFU/cell) syngeneic spleen cells (10⁶ cells/ml) which had been irradiated at 2,000 rad. After 5 days of culture, cells were harvested and used as effectors in cytotoxicity assays.

51Cr labelling and preparation of target cells. The day before the CTL assay, fibroblast target cells that had been grown to confluence in a T25 flask were labelled with 200 μCi of Na51CrO4 for 90 min in 0.8 ml of RPMI 1640 culture
medium without serum, washed three times, distributed in flat-bottom microtiter plates at 10^4 cells/well and left overnight at 37°C in 5% CO₂. The fibroblasts were washed once with RPMI 1640 and then infected with DA virus (20 to 40 PFU/cell) or Mengo virus strain vMC24 (10^4 PFU/cell) for 2 or 6 h, respectively, before the addition of effector cells. In other experiments, peptide VP2118-131 (10^4 PFU/cell) or Mengo virus strain vMC24 (10 PFU/cell) for 2 or 6 h, respectively, were washed once with RPMI 1640 and then infected with DA virus (20 to 40 PFU/cell). Noninfected target cells were used as negative controls (open symbols).

**CTL assay.** Cytolytic activity of restimulated CTLs was measured by a standard 51Cr release assay. Serial dilutions of effector cells were set up in triplicate and added to 200 μl of medium to the prepared target cells at the indicated ratios. After incubation for 4 h, plates were centrifuged and 51Cr release was measured. Spontaneous release was determined by using the following equation: [sample release – spontaneous release]/[maximum release – spontaneous release] × 100. Maximum spontaneous release values were always <5% of total release values. LDA. Precursor frequencies of TMEV-specific CTLs were estimated by limiting-dilution analysis (LDA). Responder spleen cells derived from DA virus-infected C57BL/6 mice were cultured at 800 to 50,000 spleen cells per well in 24 replicates per each concentration responder cell in round-bottom microtiter plates. Stimulator cells generated by infecting syngeneic spleen cells with DA virus were irradiated and added at a concentration of 3 × 10^5 cells/well to each well in RPMI 1640 culture medium supplemented with 5% interleukin 2-conditioned medium, as previously described (4). On day 7 of culture, each well was split and assayed for cytolytic activity on 31Cr-labelled, DA virus-infected or peptide VP2118-131-loaded C57SV fibroblasts (10^4 cells/well). Wells were considered positive when the 51Cr release exceeded the average spontaneous 51Cr release of control wells plus 3 standard deviations. The frequencies were determined by the zero-order term of the Poisson distribution. Data from individual experiments were compatible with the hypothesis of single-hit kinetics, and lines were fitted by the least-squares method (16). Precursor estimates are given with 95% confidence limits.

**RESULTS**

**CTL activity in TMEV-infected C57BL/6 mice.** In order to analyze the CTL response to TMEV infection in resistant mice, C57BL/6 mice were inoculated intraperitoneally with 10^6 PFU of DA virus. In vitro-restimulated splenocytes were prepared as described in Materials and Methods following a protocol developed for the detection of Mengo virus-specific CTLs (12). These splenocytes clearly showed cytotoxicity against syngeneic DA virus-infected C57SV fibroblasts (Fig. 2A). No cytolytic activity was observed on uninfected target cells or on allogeneic DA virus-infected KSSV target cells (Fig. 2A). Thus, cytotoxicity obtained from DA virus-infected C57BL/6 mice was virus specific and MHC restricted. Depletion of CD8+ T cells resulted in a drastic reduction of specific lysis, whereas depletion of CD4+ T cells did not reduce the lysis of DA virus-infected target cells (data not shown). Therefore, the cytotoxic activity detected is mediated by classical CD8+ CTLs that are likely to be MHC class I restricted. To determine the MHC class I restriction of CTLs induced by DA virus in C57BL/6 mice, DA virus-infected or uninfected L cells (H-2k) expressing either the H-2k* or H-2k+ molecules were used as target cells. As shown in Fig. 2B, the cytolytic activity of DA virus-induced CTLs was predominantly restricted to the H-2k* molecule. A minor cytotoxic activity was restricted to the H-2k+ molecule. These findings are consistent with previous data showing that resistance to TMEV infection maps to the H-2D locus (5, 6).

**CTLs derived from Mengo or DA virus-infected C57BL/6 mice are cross-reactive.** We have recently identified MHC class I-restricted epitopes for Mengo virus-induced CTLs. Two H-2Dk-restricted epitopes are located in the VP2 capsid protein. One of them, designated VP2122-130, is conserved among the VP2 capsid proteins of Mengo virus and of all TMEV strains (12a). Therefore, we tested Mengo virus-induced CTLs for their cytolytic activity on syngeneic, DA virus-infected target cells (Fig. 3A). The cytolytic activity of Mengo virus-induced CTLs against DA virus-infected target cells was as strong as that against homologous Mengo virus-infected target cells. This suggested that the conserved viral peptide is presented on both Mengo and DA virus-infected target cells. Reciprocally, we tested the cytolytic activity of DA virus-induced CTLs on Mengo virus-infected target cells (Fig. 3B). DA and Mengo virus-induced CTLs showed the same cytolytic activity against Mengo virus-infected target cells. Taken together, these data show that Mengo and DA virus-induced CTLs are cross-reactive at the level of both effector and target cell recognition.

Furthermore, when syngeneic C57SV fibroblasts loaded with the VP2118-131 peptide were used as target cells for DA virus-induced CTLs, the specific lysis of peptide-loaded target cells was even higher than that of DA virus-infected target cells (Fig. 4). Thus, the cross-reactivity between Mengo and DA virus-specific CTLs is mainly directed against the VP2122-130 epitope.

The CTL response against VP2122-130 of DA virus-infected C57BL/6 mice is immunodominant. The high cytolytic activity of DA virus-induced CTLs against peptide VP2118-131-loaded C57SV fibroblasts (Fig. 2A) indicates that the cross-reactivity between Mengo and DA virus-specific CTLs is mainly directed against the VP2122-130 epitope.
target cells suggested that the CTL response against this peptide may be immunodominant in C57BL/6 mice. To examine this point, LDAs were performed as described in Materials and Methods. After 7 days of culture, each well was assayed for specific cytolytic activity on DA virus-infected or peptide-loaded target cells and specific CTL precursor (CTLp) frequencies were determined (Fig. 5). CTLp frequencies in DA virus-infected C57BL/6 mice against DA virus-infected or peptide VP2118-131-loaded target cells were approximately the same (1 in 11,000 and 1 in 13,000, respectively), suggesting that the VP2122-130 epitope is indeed immunodominant. Although the CTL frequencies varied somewhat between individual limiting-dilution experiments, the ratio of DA virus to VP2122-130-specific CTLp remained unaltered (data not shown). The possible immunodominance of the VP2122-130 epitope was further investigated by split-well analysis (Fig. 6). Thus, wells which were positive in the LDAs and which contained fewer cells than the estimated CTLp frequency, i.e., wells which were likely to contain cells derived from a single TMEV-specific CTLp, were analyzed for their cytotoxicity on DA virus-infected and peptide-loaded target cells. The results showed that 73% of positive wells recognized both target cells. Thus, our results demonstrate that the vast majority of DA virus-induced CTLps are directed against a single viral epitope, VP2122-130.

DISCUSSION

Although CTL responses in the course of picornavirus infections have been studied for some years, the characterization of CTL epitopes for this family of viruses is still scanty. For example, it was shown that in B10 mice (H-2b) infected with the DA strain of TMEV, CTLs are directed at the VP1 and VP2 capsid proteins. However, the peptides responsible for these responses were not identified (17). We showed that the Mengo virus-specific CTL response in C57BL/6 (H-2b) mice is directed predominantly against the VP2 capsid protein (12). More recently, we defined two dominant, H-2Dk-restricted epitopes in this protein: VP2122-130 and VP2146-154 (12a). In the present study, we demonstrated that the serologically distinct DA and Mengo viruses share an immunodominant, H-2Dk-restricted CTL epitope within amino acid residues 122 to 130 of the VP2 capsid protein, indicating identical, or very similar, antigen processing and MHC class I binding for both viruses. Interestingly, this peptide does not fit the consensus sequence defined by Ramensee et al. for H-2Dk-restricted epitopes (24). This emphasizes the usefulness of functional assays in the selection of peptides to construct recombinant vaccines. The CTL cross-reactivity between DA and Mengo viruses was reciprocal; i.e., CTLs induced by Mengo virus killed DA virus-infected target cells and vice versa.

Epitopes shared between closely related viruses, such as measles virus and canine distemper virus, have been identified before (3), although CTL cross-reactivity was not demonstrated. Interestingly, a nonreciprocal CTL cross-reactivity has been observed in BALB/c mice (H-2d) against an immunodominant epitope shared by two strains of lymphocytic choriomeningitis virus, showing that two viruses with slightly divergent sequences are able to induce CTLs that differ in their capacity to recognize the other strain (30). CTL cross-reactivity due to
the recognition of unrelated peptides has also been shown (7, 21, 29).

The H-2D gene is responsible for the resistance of the C57BL/6 mouse to the persistent infection by the DA strain of TMEV (6, 8, 25, 28). Therefore, the clearance of the virus by this strain of mouse is most likely due to the H-2Db-restricted CTLs characterized in this article. It is striking that clearance can be achieved by an oligoclonal CTL response against a single epitope. This might be possible in part because the epitope is located in one of the strands of the beta barrel of VP2 (14, 20). Mutations in these beta sheaths usually yield noninfectious viruses (unpublished observation). This reduces considerably the likelihood of appearance of CTL escape mutants.

Mengo virus and TMEV are closely related cardioviruses which infect primarily the CNS. Our data, together with unpublished results, suggest that for these viruses, CTLs directed against the same epitope may have opposite roles in pathogenesis. Thus, immunization of C57BL/6 mice with recombinant vaccinia virus encoding the VP2 capsid protein of Mengo virus did not completely protect against a subsequent lethal challenge with Mengo virus, in spite of a strong VP2-specific CTL response. Furthermore, depletion of CD8\(^+\) T cells prior to inoculation with Mengo virus reduced neuropathological symptoms and mortality (12a). A similar finding has been described in the case of infection by EMCV (15). On the other hand, as discussed above, CTLs directed against the same
epitope in the course of DA virus infection play a central role in viral clearance. It will be particularly instructive to study the differences in the interplay among virus replication, viral spread, and CTL response which underlie these opposite functions.

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