The Putative Natural Killer Decoy Early Gene m04 (gp34) of Murine Cytomegalovirus Encodes an Antigenic Peptide Recognized by Protective Antiviral CD8 T Cells

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Several early genes of murine cytomegalovirus (MCMV) encode proteins that mediate immune evasion by interference with the major histocompatibility complex class I (MHC-I) pathway of antigen presentation to cytolytic T lymphocytes (CTL). Specifically, the m152 gene product gp37/40 causes retention of MHC-I molecules in the endoplasmic reticulum (ER)-Golgi intermediate compartment. Lack of MHC-I on the cell surface should activate natural killer (NK) cells recognizing the “missing self.” The retention, however, is counteracted by the m04 early gene product gp34, which binds to folded MHC-I molecules in the ER and directs the complex to the cell surface. It was thus speculated that gp34 might serve to silence NK cells and thereby complete the immune evasion of MCMV. In light of these current views, we provide here results demonstrating an in vivo role for gp34 in protective antiviral immunity. We have identified an antigenic nonapeptide derived from gp34 and presented by the MHC-I molecule Dd. Besides the immunodominant immediate-early nonapeptide consisting of IE1 amino acids 168-176 (IE1168-176), the early nonapeptide m041243-251, is the second antigenic peptide described for MCMV. The primary immune response to MCMV generates significant m04-specific CD8 T-cell memory. Upon adoptive transfer into immunodeficient recipients, an m04-specific CTL line controls MCMV infection with an efficacy comparable to that of an IE1-specific CTL line. Thus, gp34 is the first noted early protein of MCMV that escapes viral immune evasion mechanisms. These data document that MCMV is held in check by a redundancy of protective CD8 T cells recognizing antigenic peptides in different phases of viral gene expression.

Cytomegalovirus (CMV) disease with severe organ manifestations is restricted to the immunocompromised or immature host (for overviews, see references 22, 36, 57, and 66). This finding implies that an intact immune system is effective in controlling CMV infection at crucial organ sites. Accordingly, the redundant molecular strategies reported for human CMV (HCMV) and murine CMV (MCMV) to interfere with the major histocompatibility complex (MHC) class I (MHC-I) pathway of antigen presentation (for an overview, see reference 19) do not result in an immune evasion of productively infected tissue cells.

MHC-I-restricted CD8 T cells are the principal antiviral effector cells in the long-term control of HCMV (56, 58) and MCMV (42, 48, 52, 54, 61; for a review, see reference 33) infections, whereas natural killer (NK) cells form the first line of defense early in the infection (3). Specifically, in vivo depletion of the CD8 subset, but not of the CD4 subset, resulted in lethal multiple-organ MCMV disease in an experimental setting of reconstitution after bone marrow transplantation (BMT) (41). Accordingly, CD8 T cells, but not CD4 T cells, isolated from pulmonary infiltrates after BMT and MCMV infection (24) were effectual in controlling MCMV in immunodeficient recipients after adoptive cell transfer (1). The nature of the viral antigenic peptides presented to CD8 T cells via the MHC-I pathway of antigen presentation is therefore an issue central to the understanding of immunity to CMV.

In the case of MCMV infection of BALB/c mice (H-2d), which express the MHC-I molecules Kd, Dd, and Ld, we (50) have demonstrated an immunodominance of intermediate-early (IE)-phase proteins in the recognition of infected target cells by CD8-expressing cytolytic T lymphocytes (CTL). Thereafter, the immunodominant antigen was mapped to exon 4 of the ie1 gene encoding the IE1 protein pp89 (10). It was identified as IE1 peptide YPHFMPTNL presented by Ld, first by systematic screening of synthetic peptides (51, 53) and finally by its identification among the peptides naturally processed in infected cells and organs (8, 16, 24). The capacity of this single peptide to induce protective immunity was documented with a recombinant vaccinia virus containing the peptide-coding 27-nucleotide sequence (9). The findings were later confirmed by the demonstration of protective immunity induced by vaccination with IE1-encoding plasmid DNA (17).

The reason for the immunodominance of the IE1 peptide remained unclear, but an attractive explanation (Fig. 1) was provided by the observation of IE1 immune evasion effective in the early (E) phase of the viral replication cycle (7, 47). Molecular analysis of the phenomenon identified redundant immune evasion mechanisms effected by E proteins (for an overview, see reference 19). Specifically, the m152 E gene product gp37/40 was identified as the viral factor responsible for the retention of peptide-loaded MHC-I complexes in the endoplasmic reticulum (ER)-Golgi intermediate compartment and cis-Golgi, thereby preventing peptide presentation to CD8 T cells (6, 63, 67); more recently, the m06 E gene product gp48 was shown to target MHC-I complexes for lysosomal degradation (55). Since these mechanisms are not peptide specific, presentation of antigenic peptides should generally be precluded in the E-phase; consequently, antigenic peptides derived from E proteins were not expected to exist. The expres-
sion of IE1 precedes expression of the E genes, a temporal advantage that might explain the immunodominance of the IE1 protein.

However, a number of long-neglected observations argue against this explanation. (i) In the original work from 1984 describing the existence of IE-phase-specific CTL among polyclonal MCMV-primed CTL, recognition of E-phase target cells as well as of late (L)-phase target cells was already apparent (49). (ii) Under conditions of IE1 immune evasion, E-phase target cells were lysed by a CTL clone that recognized an unidentified antigen specified in EcoRI fragment F of the MCMV genome and presented by Ld (7). (iii) Antiviral CTL derived from pulmonary infiltrates after BMT preferentially lysed E-phase target cells (24). Even though the IE1 nonapeptide proved to be the only antigenic peptide detected by polyclonal pulmonary CTL among naturally processed peptides derived from MCMV-infected lungs, a comparison between IE1-specific CTL activity and overall CTL activity in pulmonary infiltrates predicted the existence of a multitude of subdominant antigenic peptides invisible as individual entities (24), many of which are expected to be E-phase specific. (iv) In mixed bone marrow chimeras resulting from the transplantation of BALB/c bone marrow cells into the Ld gene deletion mutant BALB/c-H-2^dmin, a significant component of the protection against MCMV infection of the Ld-negative parenchymal tissue cells of the recipient was attributed to recipient-derived Ld-negative CD8 T cells (1). This finding clearly implied the existence of undefined antigenic peptides presented by Kd or Dd. (v) Most recently, work by Morello et al. gave evidence for protective immunity induced by the E protein specified by the M84 gene of MCMV, the positional homolog of HCMV UL84 (40). Collectively, these data indicated the existence of more than one antigenic E-phase peptide, but no such peptide has ever been identified in its amino acid sequence.

In this work we report on the identification of an antigenic peptide presented by Dd and specified by the E gene m04, known to encode gp37/40 (31). A CTL line (CTLL) specific for the m04 nonapeptide (m04-CTLL) protected against MCMV infection in vivo with an efficacy comparable to that of an IE1 nonapeptide-specific CTLL (IE1-CTLL). The m04 nonapeptide is the second antigenic peptide known for MCMV and the first antigenic E-phase peptide that apparently escapes the redundant immune evasion mechanisms of MCMV.

**MATERIALS AND METHODS**

Generation of CTLL with specificity for MCMV peptides. Immunocompetent, 8- to 10-week-old female BALB/c mice (MHC haplotype H-2^d) were sensitized by subcutaneous infection at the left hind footpad with 10^3 PFU of purified MCMV (35), strain Smith ATCC VR-194/1981. At >3 months after infection, at a time when productive infection is cleared in all organs, spleen cells were prepared according to standard protocols to serve as a source of MCMV-specific memory T cells. Throughout, cell culture was performed under standard conditions at 37°C, 5% CO2, and humidified atmosphere.

(i) Bulk culture for the generation of peptide-specific CTLL. The cultivation protocol was as used in our previous work (46), with some modification. In brief, 1.5 x 10^7 spleen cells were seeded in a volume of 2 ml in 24-well culture plates in clone medium, minimal essential medium alpha (product no. 22561-021; Gibco BRL, Eggenstein, Germany) supplemented as described elsewhere (46) except that fetal calf serum (FCS) was reduced to 7.5% (vol/vol) and the antibiotics kanamycin and amphotericin B were not routinely added. Restimulation of peptide-specific memory T cells in the spleen cell population was achieved by synthetic peptides added in a molarity optimized for each peptide. Conditions for the generation of IE1-CTLL were reported previously (24). Usually, restimulations were performed weekly by a 1:1 split and readdition of 1 ml of clone
medium containing the appropriate peptide and 100 U of recombinant human interleukin-2 (rIL-2; 2.8 × 10^5 U/ml) per ml of culture medium; generously supplied by the Sandoz Research Institute, Vienna, Austria). Monospecificity of the CTLL-2 cells (a subclone of the CTLL-1 clone) was assessed by immunoperoxidase staining with a mouse monoclonal antibody (clone 128D7; kindly provided by Dr. John Ziegler, New York, N.Y.).

(ii) Microculture system for the screening of naturally processed peptides. Peptides derived from MCMV-infected mouse embryonic fibroblasts (MEF) were fractionated by high-performance liquid chromatography (HPLC; see below). The HPLC eluate, which corresponded to the extract derived from 0.5 × 10^7 infected MEF, was loaded on the HPLC column, and peptides were eluted at a flow rate of 0.5 ml per min on a linear acetonitrile gradient (solution A, 0.1% [vol/vol] TFA; solution B, 80% [vol/vol] acetonitrile-0.09% [vol/vol] TFA). The gradient was developed as follows: min 0 to 6, solution A; min 6 to 46, linear increase of solution B from 10% to 100% solution B; min 50 to 58, decrease to 0% solution B; and min 54 to 58, solution A. A aliquots of the 0.8-ml fractions were distributed into wells of microwell plates and lyophilized for storage until used for the generation of CTLL and for pulsing of target cells in the cytolytic assays.

(ii) Synthetic peptides. Custom peptide synthesis in ca. 1 mg scale and a purity of >95% were prepared by JERINI Bio Tools GmbH (Rottweil, Germany). The synthetic peptides were dissolved in 30% (vol/vol) acetonitrile at PBS concentration of 10^{-5} M. Further dilutions were performed in medium.

(iii) IFN-γ-based ELISPOT assay. Peptide-specific CTLL derived from the spleen during acute MCMV infection (2 weeks after infection) or after the establishment of viral latency and immunological memory (>3 months after infection) were quantitated by an enzyme-linked immunospot (ELISPOT) assay detecting single CTLL T cells that secrete gamma interferon (IFN-γ) upon resequestration with specific synthetic peptides. For the principle of the assay, see reference 43; for the established protocol (39, 62) published previously (15) was used, with some further modification. The assay was performed in nylon membrane-backed 96-well microtiter plates (Biodyne B nylon 66 membrane product no. 256154; Nunc, Wiesbaden, Germany). The membrane bottom of the wells was coated with rat anti-mouse IFN-γ monoclonal antibody (clone RMMG-1; product no. AMC 4834; Biosource Europe, Rutesheim, Germany) contained in bicarbonate coating buffer (pH 9.6) at a concentration of 10 μg per ml. Surplus antibody was washed out, and the membrane was saturated with clone medium (with no IL-2) for 1 h at 37°C. Peptide-presenting cells (PPC) were added to the wells of the assay plate in a concentration of 10^5 cells per 50 μl of clone medium (with no IL-2). PPC were P815 mastocytoma cells stably transfected with human B7-1 (CD80) cDNA (2), referred to as P815-B7 cells (used with the kind permission of L. L. Lanier, DNAX, Palo Alto, Calif.), pulsed with the appropriate peptide at a final concentration of 10^{-8} M. As observed by one of us (G. Geginat, unpublished data), the use of P815-B7 instead of P815 significantly improves the specificity of the assay by enhancing the signal-to-background ratio. A solution of the peptide was washed out before use of the PPC in the ELISPOT assay. As IFN-γ secreting effector cells to be quantitated, graded numbers of spleen cells (here 10^3 and 10^4 cells) were added in 50 μl of clone medium (with no IL-2). After incubation for ca. 16 h at 37°C, cells were removed by extensive washing with PBS containing 0.25% (vol/vol) Tween 20 (PBS-Tween). Bound IFN-γ was labeled for 2 h at 20°C with biotinylated rat anti-mouse IFN-γ MAb (50 μl of 1 μg/ml; clone XMG1.2, product no. 18112D; Pharmingen, Hamburg, Germany), washed with PBS-Tween, and then incubated for 2 h at 20°C with horseradish peroxidase-streptavidin conjugate (50 μl of 4 μg/ml; Dianova, Dusseldorf, Germany). After further washing, 50 μl of aminoethylcarbazole solution (15) was added as the substrate, yielding a brown precipitate. After 5 to 10 min, the membranes were washed with PBS to remove nonbound substrate, representing individual peptide-specific cells, were counted under a stereomicroscope.

Cytofluorometric detection of intracellular IFN-γ. The functional capacity of CTLL to produce IFN-γ upon stimulation with the specific peptide was tested by the cytofluorometric staining of intracellular IFN-γ according to an established protocol (43; for the principle of the assay, see reference 21), with some modification. Peptide-specific CTLL were seeded in 96-well round-bottom microtiter plates at a concentration of 1 × 10^4 cells per well (product no. 31870-025; Gibco-BRL) supplemented with 5% [vol/vol] FCS, 50 μM 2-mercaptoethanol, 10 mM HEPES, 50 μU of rIL-2 per ml, stimulating peptide or peptide control at a concentration of 10^{-6} M, and 10 μg of brefeldin A (Sigma) per 100 μl. After 48 h, the cells were fixed. Harvested cells were pooled, and aliquots of 4 × 10^6 cells were resuspended in 50 μl of permeabilization solution for 30 min on ice. After washing twice with 500 μl of PBS supplemented with 1% (wt/vol) bovine serum albumin and 0.01% (wt/vol) NaN_3, the cells were boosted for 5 min at 37°C by addition of 100 μl of PBS containing 2% (vol/vol) paraformaldehyde. After washing twice with PBS, cells were permeabilized for 10 min at ca. 20°C by resuspension in permeabilization solution, which is PBS containing 1% (wt/vol) bovine serum albumin, 0.1% (wt/vol) NaN_3, and 0.4% (wt/vol) MAb anti-FcγRII/Ill (clone 2.4G2; product no. 12314D; Pharmingen, San Diego, Calif.) was added per sample. Surface staining was performed with phycoerythrin (PE)-conjugated MAb anti-CD8α (clone 53.6.7; product no. 01045A; Pharmingen). The cells were washed twice with PBS to remove excess protein and were resuspended in 100 μl of PBS. Cell fixation was performed for 20 min at ca. 20°C by addition of 100 μl of PBS containing 2% (vol/vol) paraformaldehyde. After washing twice with PBS, cells were permeabilized for 10 min at ca. 20°C by resuspension in permeabilization solution for 30 min on ice. Fixed cells were washed twice with FACS solution (Dianova), resuspended in permeabilization solution, and analyzed immediately with a FACSort cytofluorometer (Becton Dickinson, San Jose, Calif.). Data were processed by using CellQuest software (Becton Dickinson).
MCMV. Under these conditions, all mice die of multiple organ histopathology (54) and bone marrow aplasia (57) between days 10 and 18 after infection unless they receive protective T cells. CTL were transferred intravenously 2 h before infection. Survival was assessed between 50 and 70°C. After the infection was achieved by centrifugally enforced virus penetration (54).

Quantitation of virus infection and T-cell infiltration in tissues. (i) Determination of virus titers. Infectious virus was quantitated in organ homogenates by a plaque assay performed on subconfluent second-passage MEF monolayers under conditions of centrifugal enhancement of infectivity in 48-well culture plates as described previously (54). The virus titers represent the amount of infectious virus per organ and are expressed as PFU* to indicate the ca. 20-fold enhancement of infectivity (25, 35) achieved by the centrifugally enforced virus penetration (54).

(ii) Quantitative two-color immunohistostaining. Livers were fixed with PBS (pH 7.4) containing 4% (vol/vol) formalin. The tissue was then processed for paraffin embedding. Sections of 2 μm were dewaxed with xylene and subjected to two-color immunohistochemistry with hematoxylin counterstaining precisely as described previously (1, 24). In essence, liver-infiltrating T cells were visualized by membrane staining with a rat MAb directed against murine CD3ε, biotinylated anti-rat antibody, and the avidin-biotin-peroxidase complex with diaminobenzidine tetrahydrochloride as the substrate. The staining was enhanced with nickel sulfate hexahydrate, yielding a black precipitate. Infectected hepatocytes were visualized by detection of intranuclear viral IE1 protein with MAb CROMA 101 (kindly provided by S. Jonjic, Medical Faculty of the University of Rijeka, Rijeka, Croatia), goat anti-mouse immunoglobulin antibody (no. M5099: Sigma), and the alkaline phosphatase-anti-alkaline phosphatase complex with new fuchsin as the substrate, yielding a brilliant red precipitate. Liver-infiltrating T cells and infected hepatocytes were counted for representative 10-mm² areas of tissue sections.

Analysis of viral gene expression. The kinetics of viral gene expression in infected MEF monolayer cultures (10-cm petri dishes with ca. 2 × 10⁶ cells per dish) was assessed by reverse transcriptase (RT)-mediated PCR (RT-PCR).

Specifically, MEF were infected with purified MCMV in the third cell culture passage at a multiplicity of infection (m.o.i.) of 5-10pfu/cell. Under these conditions, >90% of the cells were infected as assessed by immunofluorescence specific for the intranuclear IE1 protein (p152) (50). The extraction buffer of a QuickPrep-Micro mRNA purification kit (Pharmacia Biotech) was added to the MEF monolayers, and detachment of cells was facilitated by using a cell scraper. Poly(A)+ RNA was purified on the basis of oligo(dT)-cellulose affinity.

(iii) RT reaction treatments prior to RT-PCR. For genes with an exon-intron structure, such as MCMV ie1 (29), primers were placed in neighboring exons, yielding size-different amplified from mRNA and contaminating DNA. In the case of all other viral genes, contaminating DNA may result in false-positive signals for gene expression and must be digested before RT-PCR. DNase treatment of poly(A)+ RNA samples was performed with RNase-free DNase (product no. M6101; Promega, Madison, Wis.). The reaction was performed for 30 min at 37°C in a volume of 10 μl with 1 μg of poly(A)+ RNA adjusted to 8 μl with RNase-free water (24). 1 μl of a 10-fold-concentrated reaction buffer (400 mM Tris-HCl [pH 8.0], 100 mM MgSO₄, 10 mM CaCl₂, and 1 μl containing 1 U of the RNase-free DNase. The digestion was stopped by inactivating the DNase with 1 μl of stop solution (20 mM EDTA, 100 mM Tris-HCl, pH 8.0) and phenol/chloroform extraction for 1 min at 65°C. The clean RNA was diluted 1:50 in RNase-free water to reduce the Mg²⁺ concentration that would otherwise affect the RT reaction. Aliquots of 10 μl were subjected to RT-PCR.

(ii) Design of primers and probes for RT-PCR. Throughout, oligo(dT) priming was used for the RT reactions. Forward and reverse primers for the detection of hprt (hypoxanthine phosphoribosyltransferase mRNA) (32) were 5′-GGGGGATACAGCCGCACTTGGTG-3′ and 5′-GGCATAGTAGGGCGCAAATTTG-3′, respectively. The 163-bp hprt amplified previously detected with the internal oligonucleotide probe 5′-GGTGTGGATATGCCCTTGAC-3′. Positions of and probes for viral gene expression refer to the genomic sequence of the Smith strain of MCMV (45; GenBank accession no. M56829 [complete genome]). For detection of ie1 cDNA, forward primer 5′-181750-181776-3′ (5′-CCGATGCAATACGATATCGACCG-3′) and reverse primer 5′-181374-181399-3′ (5′-GCGAGCTGTGGTGGAACCTGGAC-3′) were used. The 280-bp ie1 amplified by probe 5′-181504-181531-3′ (5′-GCCCTGATCCCTGAGGATCCG-3′) directed against the exon 3-exon splicing junction. m04 cDNA was detected with forward primer 5′-3423-3446-3′ (5′-GGCATAACCAGACTGGATCCTGGC-3′) and reverse primer 5′-3746-3723-3′ (5′-AGCGCTCGTGTCGTGGCTGCTTCG-3′). The 324-bp m04 amplified by probe 5′-3616-3640-3′ (5′-CAGTCCCATGACGCTGGGATATGCCCTTGAC-3′) was detected. Forward and reverse primers for detection of m152 cDNA were 5′-210043-210063-3′ (5′-GGTTAGACAGCAGCGTGGTTCG-3′) and reverse primer 5′-210026-210045-3′ (5′-CTCCGGTCCTGCCGCAGTCTCC-3′), respectively. The 301-bp m152 amplified by probe 5′-210067-210097-3′ (5′-TTCGGGAGACTTGGGTCG-3′) was detected.

(iii) RT-PCR. Reactions were carried out by using an automated thermal cycler (GeneAmp PCR System 9700; Perkin-Elmer Applied Biosystems, Norwalk, Conn.). The RT reaction was performed with 10 μl of poly(A)+ RNA (ca. 10 ng, representing the yield of ca. 2 × 10⁶ infected MEF) adjusted with RT reaction buffer to a total volume of 20 μl [final concentrations: 60 mM KCl, 15 mM Tris-HCl (pH 8.4), 3 mM MgCl₂, 10 mM diethanolamide, 20% (vol/vol) glycerol, 1 mM each deoxynucleoside triphosphate, 12.5 pmol of oligo(dT) primer, 10 U of RNasin, and 100 U of Moloney murine leukemia virus RT (Gibco BRL)] for 1 min at 25°C. The reaction was stopped by heating to 95°C for 5 min, and the cDNA samples were kept on ice until PCR. For the amplification of cDNA sequences by PCR, 30 μl of PCR mix (60 mM KCl, 15 mM Tris-HCl [pH 8.4], 3 mM MgCl₂, 20% [vol/vol] glycerol, 25 pmol of each primer, and 10 μl of Taq polymerase [Eurobio, Roehrm, Germany]) was added. The PCR time-temperature profile for cycles 2 to 29 was as follows: denaturation for 30 s at 96°C, annealing for 1 min at the primer-dependent temperature (58°C for hprt and ie1 and 65°C for m04 and m152), and elongation for 1 min at 72°C. In the first cycle, denaturation was performed for 3 min at 95°C. In the last cycle (cycle 30), the elongation time was extended to 5 min. Amplification products (15 μl) were visualized by standard procedures of 2% (wt/vol) agarose gel electrophoresis, Southern blot, hybridization with the appropriate γ-3²-end-labeled oligonucleotide probe, and autoradiography.

RESULTS

Search for novel MCMV-specific antigenic peptides. In a previous report on the detection of MCMV-specific MHC-I- presented peptides by pulmonary CTL of BALB/c mice (24), the long-known immunodominant, L₁-presented IE1 peptide YPHFMPNTL (53) was the only peptide detectable in HPLC fractions derived from an extract of infected lungs. However, infected target cells were recognized in the E phase of the viral replication cycle (24), even though the IE1 peptide is not presented in the E phase (7, 47). Furthermore, compared with the overall cytolytic activity asayed by CD8-directed lysis, IE1-specific CTL apparently accounted for an only minor part of the pulmonary CTL response to MCMV (24). This finding implied the existence of further antigenic peptides that remained invisible as individual entities for polyclonal pulmonary ex vivo CTL but collectively constituted a strong immune response. It was therefore necessary to develop a strategy for making the postulated “invisible” peptides visible.

Antigenic peptides, which are involved in the CD8 T-cell response in vivo, leave their imprint in the specificity repertoire of memory T-cell populations. If an antigenic peptide that was naturally processed in MCMV-infected cells is present in an HPLC fraction of the cell extract, and if memory T cells with the specific T-cell receptor (TCR) are present in the splenic cell population of MCMV-sensitized mice, repeated restimulations with fractions derived from polyclonally HPLC-purified MCMV should yield fractions containing the antigenic peptide. Fractions of an HPLC control run performed with an extract from uninfected MEF did not select any CTL from memory spleen cells (not shown).

By contrast, restimulation of memory spleen cells with HPLC fractions derived from productively infected MEF identified several fractions with antigenic activity (Fig. 2). Most peptides eluted from the HPLC column between fractions 20 and 40 (Fig. 2A). Antigenic peptides were found to be contained most prominently in fractions 22, 23, 27, 28, and 29, and there were minor candidates as well (Fig. 2B).

When the HPLC fractions containing naturally processed MCMV peptides were used to pulse target cells for a cytolytic assay performed with IE1-CTL as effector cells, fractions 27 and 28 were positive. Likewise, IE1-CTL detected antigenic activity in fractions 27 and 28 of an HPLC separation of extract from uninfected MEF supplemented with the synthetic IE1 nonapeptide YPHFMPNTL. Thus, fractions 27 and 28 contained the already known IE1 peptide (not shown).

In conclusion, the strategy of repeated restimulation of memory T cells did not reveal numerous antigenic HPLC fractions, but fractions 22, 23, and 29 each contained at least one antigenic peptide distinct from the known IE1 peptide. Actually, this experiment was the first to clearly indicate the existence of MCMV-specific antigenic peptides in addition to the immunodominant IE1 peptide.
The MHC restriction of the thus selected HPLC fraction-specific CTLL was tested with target cells expressing any of the three MHC-I genes of the \( H^{2\text{d}} \) haplotype selectively, namely, the L-cell transfectants \( L^{-}\), \( L^{d} \), and \( L^{K} \) (Fig. 2C). It should be noted that no cytolytic activity was detected with the parental \( L^{-}\)-derived cells, which are of \( H^{2\text{a}} \) haplotype (not shown). As predicted from the known \( L^{d} \) presentation of the IE1 peptide, \( L^{d} \) restriction was seen for CTLL raised with HPLC fractions 27 and 28. Fractions 22 and 23 contained at least one peptide presented by \( D^{d} \). Fraction 29 contained at least one peptide presented by \( K^{d} \). Fractions 31 and 32, although less prominent in the assay shown in Fig. 2B, were found to contain peptides presented by \( L^{d} \). Fraction 26 revealed a promiscuous restriction, which might indicate a mixture of different peptides. The activity in fractions 34 and 42 was not confirmed by the HPLC restriction analysis. It should be noted that the pattern can vary with different memory spleen cell populations, and thus a negative result in a particular experiment does not exclude the presence of an antigenic peptide in a negative fraction. The activity in fraction 22 was most consistently observed in our experiments, and therefore we focused first on peptides in that fraction. In summary, MCMV specifies a strong and novel antigenic peptide presented by \( D^{d} \), and further candidates exist.

**D**\( ^{d} \) motif peptide library of the full-length MCMV genome. An approach to identify the peptide in fraction 22 by mass spectrometry failed due to the heterogeneity and low amount of peptides present in the HPLC-derived fraction (T. Ruppert, personal communication). Given the existence of at least one \( D^{d} \)-presented peptide (see above), we performed a screening of synthetic peptides (Fig. 3) based on Rammensee’s \( D^{d} \) binding motif (12; for an extensive overview and motif listing, see reference 44). Specifically, the nonameric \( D^{d} \) motif is \( xGpxxxxx[L, I, F] \) with MHC anchor residues glycine and proline at positions 2 and 3, respectively, and leucine, isoleucine, or phenylalanine at C-terminal position 9. The search for the motif was made for all open reading frames of the entire genome of MCMV, strain Smith, based on the complete genomic sequence published by Rawlinson et al. (45). The search revealed 35 potentially antigenic peptides proposed to be presentable by \( D^{d} \). The respective synthetic peptides were each used in three different molarities for the restimulation of MCMV-sensitized memory spleen cells. Screening for a wide range of peptide concentrations proved to be essential, because the affinity of an antigenic peptide for MHC binding is not predictable from its sequence and because suboptimal as well as supraoptimal peptide concentrations in the restimulation assay can give false-negative results (4).

With some peptides, CTLL were generated only in one culture of the triplicate. This finding is likely to reflect a very low frequency of memory T cells specific for those peptides. A prominent \( D^{d} \)-presented peptide, seen in triplicate cultures after restimulation with a peptide concentration of \( 10^{-8} \) M, matched to positions 3996 to 4022 of the MCMV genome (Fig. 3A). This sequence is located in \( Hid\text{III} \) fragment A (11) and within gene \( m04 \) encoding gp34 (31). The corresponding peptide was the sequence YGPSLYRF (aa 243 to 251) located at the junction between transmembrane region and cytoplasmic tail of gp34 (Fig. 3B).

It was an obvious question to ask in retrospect whether this \( D^{d} \)-presented synthetic peptide was indeed identical with the \( D^{d} \)-presented naturally processed peptide that had eluted in the experiment of Fig. 2 in HPLC fraction 22. Therefore, an HPLC run was performed with synthetic peptide YGPSLYRF. Surprisingly, the peptide did not elute in fraction 22 but proved to be contained in fraction 24, which was seen before (Fig. 2C) to contain a minor \( D^{d} \)-presented candidate peptide. Work is in progress to identify the fraction 22 peptide. Although fraction 24 peptide was thus discovered by serendipity, its derivation from the well-defined and particularly interesting viral NK decoy glycoprotein gp34 led us to continue with working on it.

**H**\( ^{2\text{d}} \) class I motif peptide library for gene \( m04 \) and frequencies of \( m04 \)-specific CD8 T cells. Notably, \( m04 \) contains only a single \( D^{d} \) motif. To identify further antigenic peptides processed from gp34 and presented by MHC-I molecules, memory spleen cells were repeatedly restimulated with synthetic peptides representing \( K^{d} \) and \( L^{d} \) motifs specified by the \( m04 \) gene. Specifically, the nonameric \( K^{d} \) motif is \( xY, F]xxxxx[I, L, V] \), and the nonameric \( L^{d} \) motif is \( xP, S]xxxxx[F, L, M] \) (44). The search revealed a single \( K^{d} \) motif and 11 \( L^{d} \) motifs within the
m04 open reading frame, but none of the corresponding synthetic peptides could be identified as an antigenic peptide generating short-term microculture CTLL (not shown). It is worth noting that the group of nonantigenic motifs includes an Ld motif (aa 1 to 9), which is located in the signal sequence, and were thus potentially independent of proteasomal processing (18, 64).

As a second approach for the identification of relevant antigenic peptides, the 13 synthetic peptides of the H-2d motif library of m04 (Fig. 4A) were used to stimulate acutely primed and memory spleen cells in an ELISPOT assay based on IFN-γ secretion by activated CD8 T cells (Fig. 4B and C). In accordance with the results of the CTLL microcultures, the Dd-presented peptide consisting of aa 243 to 251 (peptide 243-251) proved to be the only m04-encoded peptide that gave a reproducible and significant signal with acutely primed spleen cells (Fig. 4B) as well as with two independent memory spleen cell pools (Fig. 4B and C). Unprimed spleen cells gave no signal above the assay background (Fig. 4C) that was defined with all components except peptide. f-m04, the frequency of pool 1 memory cells (Fig. 4B) specific for the m04 peptide 243-251 (hereafter referred to as m04 peptide), was 1 per 14,000 spleen cells. As a positive reference, the ELISPOT assays were all also performed with the IE1 peptide 168-176, i.e., peptide YPHFMPTNL presented by Ld (53). The frequency of pool 1 memory cells specific for the IE1 peptide (f-IE1) was found to be 1 per 700 spleen cells. Since the proportion of CD8 T cells in the spleen is ca. 10% (varying between 8 and 12% in memory spleen cell populations), corrected frequencies f-m04 and f-IE1 are 1 per 1,400 and 1 per 70 CD8 T cells, respectively. Altogether, these assays have impressively reconfirmed the previously noted relative immu-

![Diagram](image-url)

**FIG. 3.** Identification of an antigenic peptide by screening of a Dd motif peptide library of MCMV. (A) A search for the Dd binding motif xGPxxxx[L, I, F] (44) was performed for all open reading frames of the full-length genomic sequence of MCMV (45), and corresponding synthetic nonapeptides were used at the indicated concentrations for the generation of short-term microculture CTLL by repeated restimulation of MCMV-specific memory spleen cells. The cytolytic assay was performed with P815 target cells. Data represent the cytolytic activity in individual microcultures. The genomic positions of the nonapeptide-coding sequences are given by the positions of the first nucleotides according to the listing by Rawlinson et al. (45). C, complementary strand. (B) Map of the location of the prominent antigenic peptide (nucleotide [n] positions 3996 to 4022), representing aa 243 to 251 of gp34 encoded by gene m04 (31). TM, transmembrane region; CY, cytoplasmic tail. The peptide sequence is given in one-letter code.
presentation of the IE1 peptide may prime CD8 T cells before evasna mechanisms become effectual. This plausible explanation for antigenicity might apply also to other peptides presented before the expression of gp37/40. We therefore studied the kinetics of MCMV gene expression in infected MEF by RT-PCR (Fig. 5). Consistent with previous findings (28), ie1 gene expression was apparent at 1.5 h postinfection and the IE1-specific poly(A)$^+$ RNA remained detectable throughout the viral replication cycle, with a phosphonoacetic acid (PAA)-sensitive reexpression in the L phase (47). In agreement with a previous study (67), expression of m152 was seen early in the E phase, with a maximum of m152-specific poly(A)$^+$ RNA detectable at 4 h postinfection, declining thereafter. Expression of the E-phase gene m04 encoding gp34 clearly occurs later in the E phase. Most importantly, Kleijnen et al. have previously shown that expression of m152 precedes expression of m04 also on the protein level (31). We could show here PAA-sensitive reexpression of m04 in the L phase. One may therefore conclude that presentation of the m04 peptide occurs after rather than before the MHC-I retention mediated by m152-encoded gp37/40.

**Establishment of an m04-CTLL.** An analysis of the properties of m04-specific CTL requires their large-scale propagation. CTLL were therefore generated in bulk cultures by stimulation of MCMV-sensitized memory spleen cells with the synthetic m04 peptide YGPSLYRRF (Fig. 6). We document here the development of the cultures during three rounds of restimulation (Fig. 6) to emphasize the crucial importance of the peptide concentration used for restimulation. It may be considered trivial that low concentrations of the stimulating peptide, in this case $10^{-10}$ M, can fail to provide the required stimulation. Even though not without precedent (4), it is still less well known that generation of CTLL can also fail because of supraoptimal peptide concentration. Thus, at $10^{-7}$ M m04 peptide used for restimulation, CTL activity vanished after three restimations. The optimal condition for the generation of an m04-CTLL proved to be restimulation with $10^{-8}$ M peptide, and plateau lysis in the cytolytic assay was reached when target cells were pulsed with $10^{-8}$ to $10^{-7}$ M peptide.

**Functional activity in vitro: comparison between IE1-CTLL and m04-CTLL.** If we wish to compare antiviral functions of nodominance of the IE1 peptide (24, 50), but the m04 peptide was clearly identified as a second antigenic peptide of quantitative significance in the in vivo CDS T-cell response and generation of immunological memory to MCMV.

**Expression of the immune evasion gene m152 precedes the expression of m04.** The m152 E-phase gene product gp37/40 prevents presentation of antigenic peptides by retention of peptide-loaded MHC-I complexes in the ER-Golgi intermediate compartment and cis-Golgi (67). Expression of the IE-phase gene ie1 precedes expression of m152, and therefore
CTLL with different peptide specificities, it is of interest to know the requirements for triggering an effector function. The affinities of the interactions between peptide and presenting MHC molecule and between TCR and MHC-peptide complex are determinants of the amount of peptide that needs to be processed and presented in infected cells in order to elicit antiviral effector function. An IE1-CTLL, generated from memory spleen cells by restimulation with $10^{-2} \text{M}$ IE1 peptide YPHFMPTNL, required 100-fold less peptide for cytolytic activity (Fig. 7A, left). Specifically, half-maximal lysis of peptide-pulsed target cells occurred at $10^{-11} \text{M}$ and plateau lysis occurred at $10^{-9} \text{M}$, compared with $10^{-9}$ and $10^{-7} \text{M}$, respectively, required with the m04-CTLL. Furthermore, even at the optimal conditions for target cell formation, the IE1-CTL were superior in terms of cytolytic potential (Fig. 7A, right).

Expression and secretion of IFN-$\gamma$ upon sensitization with MHC-presented peptide is another important effector function of CD8 T cells. Both CTLL were capable of expressing IFN-$\gamma$ after stimulation with a saturating concentration of their corresponding peptides (Fig. 7B). Neither of the two CTLL expressed IFN-$\gamma$ spontaneously, and the induction of IFN-$\gamma$ required recognition of the specific MHC-peptide complex.

In conclusion, both CTLL exert effector functions when their specific peptide is adequately presented, but the IE1-CTL requires less peptide for its effector function.

Control of MCMV infection in vivo: comparison between IE1-CTLL and m04-CTLL. Gene $m04$ is expressed in the E phase (31) and reexpressed in the L phase of the viral replication cycle (Fig. 5). It was therefore obvious to examine whether infected target cells are lysed by m04-CTL in the E phase and/or in the L phase. The assay was performed, as usual in this field, with MEF infected in vitro. To our surprise, the result was negative (not shown). Admittedly, this was at first a

**FIG. 6. Generation of m04-CTLL.** Memory spleen cells derived from BALB/c mice at 4 months after infection with MCMV were restimulated under bulk culture conditions with synthetic m04 peptide YGPSLYRRF at the molar concentrations indicated. Panels A to C show cytolytic activity of CTLL after one to three rounds of restimulation, respectively. The cytolytic assay was performed at an E/T ratio of 15:1. Target cells were P815 mastocytoma cells pulsed with the m04 peptide at the molar concentrations indicated.

**FIG. 7. Properties of m04-CTLL and IE1-CTLL in vitro.** (A) Comparison of cytolytic effector function. (Left) Peptide dose dependence of target cell recognition. Target cells were P815 mastocytoma cells pulsed with the indicated molar concentrations of the appropriate synthetic peptide. The assays were performed at an E/T cell ratio of 15:1. The 100-fold peptide molarity difference between the two CTLL is highlighted by a two-headed arrow. (Right) Cytolytic potential of the two CTLL, compared by E/T titration. P815 mastocytoma target cells were pulsed with optimal molar concentrations of synthetic peptide, $10^{-7}$ and $10^{-8} \text{M}$ for m04-CTLL and IE1-CTLL, respectively. (B) Detection of intracellular IFN-$\gamma$ by two-color cytofluorometric analysis. Production of IFN-$\gamma$ was stimulated in IE1-CTLL and m04-CTLL by sensitization with $10^{-6} \text{M}$ synthetic peptide, with the heterologous peptides serving as negative controls. CTL were stained for the expression of IFN-$\gamma$ (fluorescein fluorescence [FL-1]; abscissa) and CD8 (PE fluorescence, FL-2; ordinate). Quadrants (dotted lines) were defined by omission of PE-conjugated specific antibody in the case of CD8 and by a fluorescein-conjugated isotype control antibody in the case of IFN-$\gamma$. All cells of both CTLL expressed CD8. Data obtained with 25,000 and 12,500 cells for IE1-CTLL and m04-CTLL, respectively, are shown as contour plots in a 50% log mode.
disappointment. We therefore addressed the possibility that the m04 peptide might result from gp34 processing after exogenous loading of the MHC-I pathway following virus penetration (for a review, see reference 26) rather than in the course of viral gene expression. Such a mechanism was described previously by Reddehase et al. for structural proteins of MCMV (49, 50) and appears to apply also to the processing of the HCMV tegument protein pp65 (38, 65). However, even at a multiplicity of infection of 100 carried out under inhibition of viral gene expression (49), the m04 peptide was not presented (not shown). We therefore concluded that the antigenicity of gp34 does not result from exogenous loading of the MHC-I pathway of antigen processing and presentation.

Moreover, the m04 peptide was not detected with m04-CTLL among naturally processed peptides isolated from MEF during the E phase (not shown). These in vitro data clearly contrast with the fact that m04/gp34-specific memory cells were generated during in vivo infection at a frequency of ca. 1 in 1,400 CD8 T cells and that CTLL with that specificity were so easily retrieved from memory spleen cell populations. Apparently, infected tissue cells in vivo must have adequately processed and presented this peptide, and results obtained with MEF in vitro are thus prone to mislead most likely because MEF are not representative of the cell types relevant to infection in vivo. To make the point clear, we tested the in vivo antiviral potential of the two CTLL in comparison by adoptive transfer into immunosuppressed, lethally infected recipients. Virus infection and T-cell infiltration of the liver were assessed by quantitative two-color immunohistology, simultaneously visualizing infected hepatocytes by red-stained intranuclear IE1 protein and infiltrating CTLL by black-stained CD3ε surface molecules (Fig. 8). In the absence of CTLL transfer, only few residual liver T cells were present (Fig. 8B) and failed to control the infection, resulting in plague-like, necrotic foci of infection in liver parenchyma with no signs of inflammation (Fig. 8A, panel a for overview and panel a* for detail). Adoptively transferred m04-CTLL controlled the infection in a dose-dependent manner (Fig. 8A, panels b to d for overview and b* to d* for detail). Notably, the infiltration of infected tissue is not random, but the transferred CTLL are specifically attracted by infected cells, thus forming inflammatory foci (best seen in Fig. 8A, c and c*). After transfer of 10^5 m04-CTLL, the infection was resolved, and CTLL were found in clusters (Fig. 8A, d and d*). Transfer of IE1-CTL gave essentially the same histological images (therefore not shown). Quantitation of the data indicates a somewhat higher efficacy of IE1-CTL, in particular at low cell numbers (Fig. 8B).

The antiviral function of the two CTLL was not restricted to liver tissue. Both controlled the infection, as measured by virus titers, also in other organs relevant to CMV disease, such as the spleen, lungs, and adrenal glands (Fig. 9). The minor advantage of IE1-CTL over m04-CTL, as discussed above for the liver, holds true also for the spleen, whereas m04-CTL were slightly more effectual in the lungs and adrenal glands.

Our general conclusion from this set of experiments is that both CTLL were more or less equally efficient in controlling MCMV infection in a variety of organs. Altogether, MCMV appears to be held in check by a reudance of protective CD8 T cells differing in their peptide specificities. Notably, in spite of immune evasion mechanisms operative in the E phase of the viral replication cycle, a peptide derived from the E-phase protein gp34 mediates antiviral protection.

**DISCUSSION**

Recent research on CMV immunology has focused on strategies developed by HCMV and MCMV to evade recognition of infected cells by CD8 T cells and NK cells (for a review, see reference 19). While the precise molecular mechanisms of immune evasion differ somewhat between these two viruses, they conform to the general concept that genes expressed in the E phase code for proteins that interfere at various steps with the MHC-I pathway of antigen processing and presentation. Redundance in this function indicates a biological relevance from the viewpoint of the virus but also implies a leakiness inherent to each individual molecular mechanism involved.

In the specific case of MCMV, the IE-phase gene iel codes for an antigenic peptide that is immunodominant in the H-2^d haplotype (24, 50). Since its molecular identification in 1989 (53), this IE1 nonapeptide had remained the only antigenic peptide known for MCMV. The reason for its immunodominance may lie in the fact that it is processed and presented before immune evasion mechanisms become effectual (Fig. 1, left). The E-phase gene m152 encodes gp37/40, which mediates retention of peptide-loaded MHC-I complexes in the ER-Golgi intermediate compartment and cis-Golgi (63, 67) (Fig. 1, right). Another E-phase gene, m06, codes for gp48, which targets MHC-I complexes for lysosomal degradation (55). As a consequence, both mechanisms in concert should prevent the presentation of antigenic peptides to CD8 T cells and simultaneously should reduce the expression of MHC-I on the cell surface.

In light of these findings, one may see no reason to search for antigenic peptides generated and presented in the E phase or L phase. However, there are a number of observations (listed in the introduction) that suggest the existence of further antigenic peptides of MCMV. Previous work has shown a prevention of IE1 peptide presentation in the E phase (7, 47), whereas the same E-phase target cells were lysed by a CTL clone, then named clone E1, which recognized an antigen presented by L^k and specified by EcoRI fragment F (7). An early finding was set aside when the later discovery of MHC-I retention predicted a more general inhibition of presentation in the E phase (6). It is worth noting that the E1 clone, originally isolated by Del Val et al. (7), still exists and was recently revived in our lab. We could reproduce the earlier results showing that clone E1 recognizes E- and L-phase target cells but not IE-phase target cells (not shown). However, despite all indirect evidence, no antigenic E-phase peptide was ever identified. It was therefore high time to replace evidence by proof. We have here identified the first antigenic peptide of MCMV that is derived from an E-phase protein.

Our search for novel antigenic peptides of MCMV was free from any supposition regarding candidate proteins, including gp34. It was therefore more than a surprise when a gp34-derived peptide was identified. Owing to the careful work by Kleijnen et al. (31), gp34 is well characterized. It is encoded by gene m04 and is expressed late in the E phase, clearly after the expression of the MHC-I retention protein gp37/40 (31, 67). Particular interest in gp34 arose from the finding that it is complexed with MHC-I molecules at the cell surface. Notably, in the H-2^d haplotype, gp34 was found to coprecipitate with D^d and L^d but not with K^b. It was concluded that gp34 binds to newly synthesized MHC-I in the ER, transports the complex to the cell surface, and thereby restores MHC-I surface expression in cells in which the presentation of antigenic peptides is prevented by retention of peptide-loaded MHC-I (Fig. 10, left). Since cells in the E phase are not recognized by L^d...
restricted IE1-specific CTL (7, 47), it is apparent that the MHC-I surface transport mediated by gp34 does not rescue already retained IE1-peptide–MHC-I complexes. Kleijnen et al. (31) favored the interpretation that gp34 serves to prevent the attack by NK cells, which would otherwise recognize the lack of MHC-I surface expression as missing self (23, 27). Thus, similar to the MHC-I homolog of MCMV that is encoded by gene m144 (13), gp34 is proposed to contribute to immune evasion of MCMV through interference with NK cell-mediated clearance.

FIG. 8. Control of hepatic MCMV infection by liver-infiltrating CTL. (A) Two-color immunohistological analysis visualizing infected hepatocytes (red intranuclear staining) and liver-infiltrating CD3ε-expressing T cells (black membrane staining). The indicated cell numbers of m04-specific CTL (from the same CTLL as characterized in Fig. 7) were adoptively transferred into immunocompromised, infected BALB/c recipients. ∅, control recipients with no cell transfer. The analysis was performed on day 12 after infection and cell transfer. (a to d) Overviews. The arrows point to sites of interest shown enlarged in corresponding panels (a* to d*). Bar markers represent 50 μm. (B) Quantitative two-color immunohistology after adoptive transfer of m04-CTLL (left, corresponding to panel A) or of IE1-CTLL (right). The numbers of infected hepatocytes (red dots) and of infiltrating T lymphocytes (black dots) refer to representative 10-mm² areas of liver tissue sections. Each dot represents an individual transfer recipient. Median values are marked by a horizontal bar.
recognition of this complex by specific CD8 T cells (31). How-
to a peptide-presenting MHC-I molecule may interfere with
processing and accounts for an antigenic peptide, m04243-251,
right) by demonstrating that gp34 is by itself subject of antigen
detection limit of the plaque assay.
putative role for gp34 in silencing NK cells. However, our data
have taught us the valuable lesson that assessing peptide pre-
lar enhancement of infectivity. Each dot represents an individual transfer re-
versus the IE1-CTLL (right). IE1-CTL (left) or of IE1-CTL (right).
A problem to be considered is the possibility that a structural
viral protein may be processed after virion penetration by exogenous loading of the MHC-I pathway (reviewed in refer-
gene deletion mutant. However, since deletion of m04 also eliminates the gp34-derived peptide, per-
and the presentation of a gp34-derived peptide. If native gp34
does indeed aid the presentation of a peptide derived from its
infection with MCMV had generated signif-
showed). At present, we can only speculate that the sensitivity of
preparation used for infection may include membranes of infected cells carrying gp34–MHC-I
complexes. However, when we used extremely high doses of
impression that there is any special link between native gp34
and the presentation of a MCMV m04 gene deletion mutant. However, since deletion of m04 also eliminates the gp34-derived peptide, per-
tin vitro findings, one would not propose a role for an m04 peptide
ing in protective immunity to MCMV. The in vivo data, therefore,
for antigen presentation in the E phase as a transporter for pep-
to intentionally load the alternative MHC-I pathway, includ-
ing membranes of infected cells carrying gp34–MHC-I
complexes. However, when we used extremely high doses of
observable recognition of L-phase target cells by IE1-specific
CTL (7, 47), because the ie1 gene was found to be reexpressed
L-phase target cells by IE1-specific
CTL (7, 47), because the ie1 gene was found to be reexpressed
and the presentation of a gp34-derived peptide. If native gp34
imposed with an MCMV
peptide-loaded MHC-I molecules could theoretically be tested
for antigen presentation in the E phase as a transporter for
peptide–MHC-I complexes to the cell surface to prevent NK cell recognition and simul-
mechanism has been described for MCMV (49), and CTLL that recognized structural antigens of
MCMV processed and presented in absence of viral gene ex-
expression were then established (46). The question of whether
gp34 is a virion protein of MCMV was not specifically ad-
dressed in the work by Kleijnen et al. (31). Even if it is not a
structural protein, virion preparations used for infection may
include membranes of infected cells carrying gp34–MHC-I
complexes. However, when we used extremely high doses of
virions to intentionally load the alternative MHC-I pathway,
the cells were not recognized by the gp34-specific CTL (not
shown). We therefore see no reason to assume that gp34 is
processed along this pathway.
To our surprise, in vitro-infected MEF were not lysed by
gp34-specific CTL in either the E phase or the L phase (not
shown). At present, we can only speculate that the sensitivity of
the gp34-specific CTL was too low to detect presented pep-
tide, most likely because of the low affinity of the Dd-m04
peptide-TCR interaction, which proved to be 100-fold lower
than the affinity of the Ld-IE1 peptide-TCR interaction ob-
served for the IE1-CTLL (Fig. 7A). Based on these negative in
vitro findings, one would not propose a role for an m04 peptide
in protective immunity to MCMV. The in vivo data, therefore,
have taught us the valuable lesson that assessing peptide pre-
sentation in cultured fibroblasts is inappropriate for predicting
the in vivo relevance of an antigen. Specifically, as documented
here, the in vivo infection with MCMV had generated signifi-
cant m04 peptide-specific T-cell memory, and m04 peptide-
specific CTL controlled the infection in all tissues analyzed.
However, there was no experimental evidence to support this idea.
With the same logic, one may also speculate that gp34, while it
cannot rescue already retained peptide–MHC-I complexes,
can guide peptide-loaded, newly synthesized MHC-I com-
plexes to the cell surface to prevent NK cell recognition and simul-
aneously restore the control by CD8 T cells. Promising work is in progress to identify further antigenic peptides spec-
ified in the E phase. We therefore do not wish to convey the
impression that there is any special link between native gp34
and the presentation of a gp34-derived peptide. If native gp34
does indeed aid the presentation of a peptide derived from its
own processing, this is most likely just a fancy of nature. At the
moment, it is not known whether the MHC-I molecules that
present the gp34 peptide in the E phase are biochemically
complexed with native gp34. Whether native gp34 is needed
for antigen presentation in the E phase as a transporter for
peptide–MHC-I complexes could theoretically be tested
with an MCMV m04 gene deletion mutant. However, since deletion of m04 also eliminates the gp34-derived peptide, per-
formance of this attractive experiment must await the identi-
fication of other E-phase peptides.
Alternatively, one can also envisage the possibility that pre-
sentation in the late E phase does not require assistance by
gp34. A possible reason for an escape of peptide–MHC-I com-
plexes in the late E phase may lie in the fact that expression of
the m152 retention gene wanes in the late E phase, while m04
is still expressed. An exhaustion of available retention protein
gp37/40 might also offer an explanation for the occasionally
observed recognition of L-phase target cells by IE1-specific
CTL (7, 47), because the ie1 gene was found to be reexpressed
in the L-phase (47).
A problem to be considered is the possibility that a structural
viral protein may be processed after virion penetration by
exogenous loading of the MHC-I pathway (reviewed in refer-
ce 26). Thus, presentation of antigenic peptide may precede
the immune evasion mechanisms, even though normal expres-
sion of the protein occurs in the E or L phase of the viral
replication cycle. Such a mechanism has been described for
MCMV (49), and CTLL that recognized structural antigens of
MCMV processed and presented in absence of viral gene ex-
pression were then established (46). The question of whether
gp34 is a virion protein of MCMV was not specifically ad-
dressed in the work by Kleijnen et al. (31). Even if it is not a
structural protein, virion preparations used for infection may
include membranes of infected cells carrying gp34–MHC-I
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peptide-TCR interaction, which proved to be 100-fold lower
than the affinity of the Ld-IE1 peptide-TCR interaction ob-
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vitro findings, one would not propose a role for an m04 peptide
in protective immunity to MCMV. The in vivo data, therefore,
have taught us the valuable lesson that assessing peptide pre-
sentation in cultured fibroblasts is inappropriate for predicting
the in vivo relevance of an antigen. Specifically, as documented
here, the in vivo infection with MCMV had generated signifi-
cant m04 peptide-specific T-cell memory, and m04 peptide-
specific CTL controlled the infection in all tissues analyzed.

![FIG. 9. Control of MCMV replication in further organs relevant to CMV disease. Infectious virus in the indicated organs of immunocompromised BALB/c recipients (same experiment as in Fig. 8) was quantitated by an in vitro plaque assay performed on day 12 after adoptive transfer of the indicated cell numbers of m04-CTL (left) or of IE1-CTL (right). $\varnothing$, control recipients with no cell transfer. Virus titers are given as PFU* determined under conditions of centrifugation; resolution injurious to nature.](image-url)
This implies that unlike the fibroblasts in culture, the various infected cell types in tissues must have presented the m04 peptide in an amount adequate for eliciting T-cell effector function. Enhancement of antigenic peptide presentation by IFN-γ during inflammation in vivo may contribute to the antiviral function of CTL (16, 20). That the in vivo protective function of virus-specific T cells does not always correlate with the hierarchy of CTL responses to naturally processed viral peptides is a finding reported previously for another virus infection (14).

We have here identified the first antigenic peptide of MCMV that is derived from an E-phase protein. Is this the end of the road? Certainly not! One can predict the existence of further antigenic peptides assigned to the E phase. Specifically, Del Val et al.’s proposed peptide still awaits identification. This peptide is clearly distinct from the m04 peptide (located in EcoRI-G within HindIII-A), as it maps to a distant region of the MCMV genome, namely, to EcoRI-F within HindIII-E (7, 11), and as it is presented by L1. Unlike the m04 peptide, this undefined peptide is recognized by the E1-CTLL during the E phase in MEF (7). In addition, a second E-phase-specific CTLL isolated by Del Val et al., clone E2, is proposed to recognize a peptide that does not map to EcoRI-F. Furthermore, for MCMV strain K181, recent work by Morello et al. (40) predicts an antigenic peptide encoded by gene M84 located within HindIII-C. Finally, data shown in the present report (Fig. 2) suggest the existence of a more prominent Dd-presented peptide in HPLC fraction 22 and of a significant Kd-presented peptide in fraction 29. The latter candidate is of particular interest because, as discussed above, Kd is not complexed with gp34 in the E phase (31).

Conclusion. We have shown here that MCMV is controlled in vivo by a redundancy of CD8 T cells with specificities assigned to different phases of viral gene expression. Even though recent work by Krmpotic et al. has demonstrated an in vivo modulatory function of the immune evasion gene m152 of MCMV (34), it is apparent from the course of CMV infections that all of the sophisticated viral strategies of molecular immune evasion do not eventually prevent effectual immune control. We have identified an antigenic peptide derived from gp34, a viral E-phase glycoprotein intimately involved in the viral manipulation of the MHC-I pathway in that it assists the traffic of MHC-I molecules to the cell surface. We predict that our work will stimulate an era of CMV immunology beyond immune evasion.

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REFERENCES

1. Alterio de Goss, M., R. Holtappels, H.-P. Steffens, J. Podlech, P. Angele, L. Dreher, D. Thomas, and M. J. Reddehase. 1998. Control of cytomegalovirus in bone marrow transplantation chimeras lacking the prevailing antigen-presenting molecule in recipient tissues rests primarily on recipient-derived CD8 T cells. J. Virol. 72:7733–7744.
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2. Azuma, M., A. Cayaialb, D. Buck, J. H. Philippis, and L. L. Lanier. 1992. CD28 interaction with β7 costimulates primary allogeneic proliferative response and cytotoxicity mediated by small, resting T lymphocytes. J. Exp. Med. 175:353–360.

3. Bukowski, J. P., A. A. Woda, and R. M. Welsh. 1984. Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. J. Virol. 52:191–198.

4. Busch, D. H., and E. G. Pamer. 1998. MHC class I: pEptitude: implications for immunodominance, in vitro proliferation, and diversity of responding CTL. J. Immunol. 160:4441–4448.

5. Cochet, M., W. M. Kast, A.-M. Kummer, C. Transy, C. J. M. Melief, and P. Kourilsky. 1996. Alternative splicing in the mouse H-2K gene is not necessary for the classical K+ antigen function. Immunogenetics 24:267–274.

6. Del Val, M., H. Hengel, H. H€acker, U. Hartlau, T. Ruppert, P. Lucin, and U. H. Koszinowski. 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. J. Exp. Med. 167:729–738.

7. Del Val, M., K. M€unch, M. J. Reddehase, and U. H. Koszinowski. 1989. Presentation of CMV immediate-early antigen to cytolytic T lymphocytes is selectively prevented by viral genes expressed in the early phase. Cell 58:305–315.

8. Del Val, M., H.-J. Schlicht, T. Ruppert, M. J. Reddehase, and U. H. Koszinowski. 1991. Efficient processing of an antigenic sequence for presentation by MHC class I molecules depends on its neighboring residues in the protein. Cell 66:1145–1153.

9. Del Val, M., H.-J. Schlicht, H. Volkmann, M. Messerle, M. J. Reddehase, and U. H. Koszinowski. 1991. Protection against lethal cytomegalovirus infection by a recombinant vaccine containing a single nonameric T-cell epitope. J. Virol. 65:3641–3646.

10. Del Val, M., H. Volkmann, J. B. Rothbard, S. Jonjic, M. Messerle, J. Schiede- danz, M. J. Reddehase, and U. H. Koszinowski. 1988. Molecular basis for cytolytic T lymphocyte recognition of the murine cytomegalovirus immediate-early protein pp80. J. Virol. 62:3965–3972.

11. Ebeling, A., G. M. Keil, E. Knust, and U. H. Koszinowski. 1983. Molecular cloning and physical mapping of murine cytomegalovirus DNA. J. Virol. 47:421–433.

12. Falk, K., O. R€otschke, S. Stanovanic, G. Jung, and H.-G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 351:290–296.

13. Farrell, H. E., H. Vally, D. M. Lynch, P. Fleming, G. R. Shellam, A. A. Scalo, and N. J. Davis-Poynter. 1997. Inhibition of natural killer cells by a cytomegalovirus MHC class I homologue in vivo. Nature 386:510–514.

14. Gallimore, A., T. Dunmore, H. Hengartner, R. M. Zinkernagel, and H.-G. Rammensee. 1998. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. J. Exp. Med. 187:1647–1657.

15. Gephardt, G., U. H. Koszinowski, M. Kretschmar, S. Schenk, H. Hof, M. Lalic-M€uhlf€ather, W. Goebel, and A. Bubert. 1999. Enhancement of the Listeria monocytogenes p60-specific CD4 and CD8 T cell memory by nonpathogenic Listeria innocua. J. Immunol. 162:4781–4789.

16. Gephardt, G., T. Ruppert, H. H€enger, R. Holtappels, and U. H. Koszinowski. 1997. IFN-γ is a prerequisite for optimal antigen processing in vivo. J. Immunol. 158:3303–3310.

17. Gonzales Armas, J. C., C. S. Morello, L. D. Cranmer, and D. H. Spector. 1996. DNA immunization confers protection against murine cytomegalovi- rus infection. J. Virol. 70:7921–7928.

18. Henderson, R. A., H. Michel, K. Sakaguchi, J. Shahanowitz, E. Appella, D. F. Hunt, and V. H. Engelhard. 1992. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. Science 255:1266–1268.

19. Hengel, H., W. Brune, and U. H. Koszinowski. 1998. Immune evasion by cytomegalovirus—survival strategies of a highly adapted opportunist. Trends Microbiol. 6:190–197.

20. Hengel, H., P. Lucin, S. Jonjic, T. Ruppert, and U. H. Koszinowski. 1994. Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. J. Virol. 68:289–297.

21. Hickling, J. K. 13 October 1998, posting date. Measuring human T-lymphocyte response to the murine cytomegalovirus. II. Detection of virus-specific cytotoxic T lymphocytes. J. Med. Virol. 59:451–457.

22. Ho, M. 1995. Cytomegaloviruses, p. 1351–1364. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, New York, N.Y.

23. Higlund, P., J. Sundb€ack, M. Y. Olsson-Alheim, M. Johansson, M. Salcedo, C. Ohlen, H.-G. Ljunggren, C. L. Sentman, and K. K€arre. 1997. Host MHC class I gene control of NK-cell specificity in the mouse. Immunol. Rev. 155:11–28.

24. Holtappels, R., J. Podlech, G. Geginat, H.-P. Steffens, D. Thomas, and M. J. Reddehase. 1998. Control of murine cytomegalovirus in the lungs: relative but not absolute immunodominance of the immediate-early 1 nonapeptide during the antiviral cytotoxic T-lymphocyte response in pulmonary infiltrates. J. Virol. 72:7201–7212.
replication stage-specific antigens by separate populations of in vivo active cytolytic T lymphocyte precursors. Eur. J. Immunol. 14:56–61.

50. Reddehase, M. J., and U. H. Koszinowski. 1984. Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection. Nature 312:369–371.

51. Reddehase, M. J., and U. H. Koszinowski. 1991. Redistribution of critical major histocompatibility complex and T cell receptor-binding functions of residues in an antigenic sequence after biterminal substitution. Eur. J. Immunol. 21:1697–1701.

52. Reddehase, M. J., W. Mutter, K. Münch, H.-J. Bühring, and U. H. Koszinowski. 1987. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. J. Virol. 61:3102–3108.

53. Reddehase, M. J., J. B. Rothbard, and U. H. Koszinowski. 1989. A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes. Nature 337:651–653.

54. Reddehase, M. J., F. Weiland, K. Münch, S. Jonjic, A. Lüske, and U. H. Koszinowski. 1985. Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. J. Virol. 55:264–273.

55. Reusch, U., W. Muranyi, P. Lucin, H. G. Burgert, H. Hengel, and U. H. Koszinowski. 1988. The 89,000-Mr murine cytomegalovirus immediate-early protein stimulates c-fos expression and cellular DNA synthesis. J. Virol. 62:3341–3347.

56. Reusser, P., S. R. Riddell, J. D. Meyers, and P. D. Greenberg. 1991. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood 78:1373–1380.

57. Rötzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.-G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. Nature 348:252–254.

58. Schickedanz, J., L. Phillipson, W. Ansorge, R. Pepperkok, R. Klein, and U. H. Koszinowski. 1988. The 89,000-Mr murine cytomegalovirus immediate-early protein stimulates c-fos expression and cellular DNA synthesis. J. Virol. 62:3341–3347.

59. Steffens, H.-P., S. Kurz, R. Holtappels, and M. J. Reddehase. 1998. Preemptive CD8 T-cell immunotherapy of acute cytomegalovirus infection prevents lethal disease, limits the burden of latent viral genome, and reduces the risk of virus recurrence. J. Virol. 72:1797–1804.

60. Taguchi, T., J. R. McGhee, R. L. Coffman, K. W. Beagley, J. H. Eldridge, K. Takatsu, and H. Kyono. 1990. Detection of individual mouse splenic T cells producing IFN-γ and IL-5 using the enzyme-linked immunospot (ELISPOT) assay. J. Immunol. Methods 128:65–73.

61. Thäle, R., U. Szepan, H. Hengel, G. Geginat, P. Lucin, and U. H. Koszinowski. 1995. Identification of the mouse cytomegalovirus genomic region affecting major histocompatibility complex class I molecule transport. J. Virol. 69:6109–6115.

62. Wei, M. L., and P. Cresswell. 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. Nature 256:443–446.

63. Wills, M. R., A. J. Carmichael, K. Mynard, X. Jin, M. P. Weekes, B. Plachter, and J. G. P. Sissons. 1996. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. J. Virol. 70:7569–7579.

64. Winston, D. J., W. G. Ho, and R. E. Champlin. 1990. Cytomegalovirus infections after bone marrow transplantation. Rev. Infect. Dis. 12:S776–S792.

65. Ziegler, H., R. Thäle, P. Lucin, W. Muranyi, T. Flohr, H. Hengel, H. Farrell, W. Rawlinson, and U. H. Koszinowski. 1997. A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/cis-Golgi compartments. Immunity 6:57–66.