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The Murine Cytomegalovirus Immunomodulatory Gene *m152* Prevents Recognition of Infected Cells by M45-Specific CTL But Does Not Alter the Immunodominance of the M45-Specific CD8 T Cell Response In Vivo[1]

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Although in vitro studies have shown that herpesviruses, including murine CMV (MCMV), encode genes that interfere with the MHC class I pathway, their effects on the CTL response in vivo is unclear. We identified a D^b-restricted CTL epitope from MCMV M45 by screening an MCMV genomic library using CTL clones isolated from mice infected with MCMV lacking *m152*. Because *m152* severely inhibits CTL recognition of M45 in vitro, we questioned whether an M45-specific response would be generated in mice infected with wild-type MCMV expressing *m152*. Mice infected with wild-type MCMV or MCMVΔm152 made similar responses to the M45 Ag. Moreover, we saw no skewing of the proportion of M45-specific CD8 T cells within the total MCMV-specific response after infection with MCMV with *m152*. Despite the profound effect *m152* has on presentation of M45 in vitro, it does not affect the immunodominance of M45 in the CTL response in vivo. *The Journal of Immunology*, 2002, 169: 359–365.

Herpesviruses are characterized by their ability to establish lifelong infections of their natural hosts. All herpesviruses carefully studied so far interfere with the MHC class I Ag processing and presentation pathway (1, 2). Such interference would be expected to influence the nature of the CD8 T cell response to the virus, but this has not yet been studied. Murine CMV (MCMV)[3] is a natural herpesvirus infection of mice and thus provides a good model to study the effects of immune evasion on the host immune response. This paper investigates the effect of an immune evasion gene that profoundly inhibits presentation of a viral epitope on the immunodominance of that epitope. MCMV encodes three genes, *m4*, *m6*, and *m152*, which interfere with Ag presentation to CD8 T cells. The m4/gp34 protein binds to MHC class I molecules in the endoplasmic reticulum (ER) and on the cell surface (3–5). The m6/gp48 protein redirects class I to the lysosome for degradation (6). Finally, *m152* retains class I in the ER *cis*-Golgi intermediate compartment (7, 8). Of these three genes, *m152* appears to have the most profound effect on CTL recognition: deleting *m152* makes MCMV highly visible to CTL in ^{51}Cr release assays under conditions in which the wild-type virus is not detected (3, 9, 10).

Most CD8 T cell responses to virus infections focus on very few epitopes, even though a much larger pool of peptides is processed and presented; this phenomenon is known as immunodominance (reviewed in Ref. 11). What causes some epitopes to become immunodominant over others is poorly understood, and studies are limited for large complex viruses such as herpesviruses. We would predict that the immune response to a herpesvirus would be constrained to detect the Ags least affected by its immunomodulatory genes. In contrast, the immune response to a virus lacking immunomodulatory genes should be free to focus on more abundantly presented epitopes. As a result, we and others (11) have predicted that immunomodulatory genes would affect the immunodominance hierarchy of the CTL response.

We would expect that the Ags least affected by the immunomodulatory genes would be those that are expressed in the infected cell before expression of the immunomodulatory genes. These could be structural virion proteins introduced into the cytosol at the time of viral entry. Alternately, more abundant material should be provided by the first viral gene products synthesized. As with other herpesviruses, MCMV gene expression occurs as a regulated cascade; genes are classified as immediate early (IE), early (E), and late (L). The immunomodulatory genes *m4*, *m6*, and *m152* are all E genes. An epitope from the *IE1* gene product pp89 is recognized by a substantial number of CD8 T cells in BALB/c mice (12–14). Because pp89 is expressed before *m4*, *m6*, and *m152*, it is presumably less affected than E Ags are by these immunomodulatory genes. The pp89 Ag is presented if gene expression is limited to IE genes, but once E genes are expressed pp89 presentation is abolished due to the action of immune evasion genes (15). However, the addition of IFN-γ enables pp89-specific CTL to recognize infected cells even after E gene expression (16); in contrast, the E-specific CTL clones that we have isolated from C57BL/6 (B6) mice are not able to recognize wild-type MCMV-infected targets even with the addition of IFN-γ (3–5). The fact that inhibition of

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[3] Abbreviations used in this paper: MCMV, murine CMV; DC, dendritic cell; PAA, phosphonoacetic acid; ER, endoplasmic reticulum; ICS, intracellular cytokine staining; MEF, mouse embryo fibroblast; IE, immediate early; E, early; L, late.

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pp89 presentation is readily overcome may indicate that a significant amount of Ag presentation has already occurred before the immune evasion genes are expressed.

For many years, pp89 was the only MCMV CTL Ag identified, and in consequence it has been widely studied. However, most CTL in both BALB/c and B6 mice actually recognize E and not IE Ags (15, 17–19). Thus, paradoxically, the Ags that would seem most likely to be affected by immunomodulatory genes actually elicit the majority of the immunodominant responses. This led us to question what effect the viral immune evasion genes, specifically *m152*, which has the most profound effect, have on the immunodominance of E Ags. Until recently, no immunodominant E Ags for MCMV had been described. Three CTL epitopes from E genes (*m4*, *M83*, and *M84*) for BALB/c mice were identified by Holtappels and colleagues (20–22), but these are recognized by only a small number of CD8 T cells. Very recently, Holtappels et al. (23) identified a D^d-restricted epitope in *m164*, which is as immunodominant as pp89. There is at present no information on the effect of immunomodulatory genes on the immunodominance of these epitopes.

In the current paper we describe the use of CTL clones and a genomic expression library to identify a D^b-restricted epitope encoded by the MCMV E gene *M45*. These clones were isolated from mice infected with a mutant MCMV lacking *m152* and recognize viruses lacking *m152* but are unable to detect wild-type virus in ^{51}Cr release assays. We compared the M45-specific CTL response in mice infected with MCMV with and without *m152*. Surprisingly, the percentage of CD8 T cells recognizing M45 was similar in mice infected with wild-type virus or with viruses lacking *m152*. These data indicate that while *m152* affects M45 Ag presentation in vitro it does not affect the immunodominance of M45 in vivo.

Materials and Methods

Construction of the MCMV DNA expression libraries

Two MCMV expression libraries representing the MCMV genome were constructed using MCMV DNA that was originally cloned as *Hin*dIII fragments into pUC-based plasmids (known as plasmids A–P) (24). Library *I* was constructed using equimolar amounts of *Hin*dIII fragments A, G–K, and M–P (equivalent to 99,563 bp of the MCMV genome) purified by agarose gel electrophoresis of the respective *Hin*dIII-digested plasmids. Similarly, library *II* (equivalent to 106,408 bp of the MCMV genome) was constructed using equimolar amounts of *Hin*dIII-purified fragments B–D, F, and L. Fragment E DNA (22,749 bp) was not included in the libraries. For expression library construction, the purified *Hin*dIII fragments (1,066–33,141 bp) were further fragmented by partial restriction enzyme digestion using a combination of *Bcl*I, *Bgl*II, *Bam*HI (library *I*), or *Sau*3AI (library *II*) to generate *Bam*HI compatible ends and ligated to *Bam*HI cut pcDNA4 HisMax (Invitrogen, Carlsbad, CA). The pcDNA4 HisMax A, B, and C vectors allow expression of DNA in each reading frame by staggered insertion of DNA fragments downstream of a QBI SP163 translational enhancer. Characterization of a subset of clones showed that insert frequencies were 92 (*I*) and 83% (*II*), and the average insert sizes were 1,130 (*I*) and 530 bp (*II*). Approximately 30,000 independent clones (library *I*) and 25,000 independent clones (library *II*) were arrayed in pools of 50 clones per pool. DNA for transfections was prepared using Qiagen 96 Turbo-Prep plates (Qiagen, Valencia, CA).

Library screening

The libraries were screened using K41 cells, an SV-40 transformed H-2^b fibroblast line (gift of M. Michalek, University of Alberta, Edmonton, Alberta, Canada), that were seeded at 5000 cells/well in a 96-well plate 24 h before transfection. For transfections, 100 ng (per pool) of library DNA was mixed with OptiMem (Invitrogen) and 1 μl of the transfection reagent Fugene-6 (Roche, Basel, Switzerland) and added to plated K41 cells using standard protocols. Wells were supplemented with medium 2 h after transfection and incubated at 37°C. At 48 h, 2000 T cells/well (clones 3 and 55 derived as previously described in Ref. 3) were added to the transfected K41s. After 8 h, supernatants were collected and assayed for TNF-α by bioassay using the TNF-α-sensitive indicator cell line WEHI164/clone 20, derived (S. P. Fling, unpublished data) from WEHI164 (CRL-1751; American Type Culture Collection, Manassas, VA) that is similar to that described by Khabar et al. (25).

Epitope identification

One pool of 50 DNA clones from library *II* was confirmed to elicit a positive response by two D^b-restricted MCMV-specific CTL clones (clones 3 and 55). From the positive pool, individual bacterial clones were derived and DNA was prepared and screened as described above. Individual bacterial clones that stimulated the CTL clones were sequenced. DNA was confirmed to be MCMV sequence. Peptides were synthesized based on an algorithm of optimal D^b peptide-binding motifs (http://bimas.dcrt.nih.gov/molbio/hla_bind).

CTL line and clones

Generation and maintenance of CTL clones 3 and 55 have been previously described (3). A polyclonal M45-specific CTL line was generated by methods previously described (23). Briefly, 1.5×10^7 splenocytes from $K^{b-/-}$ mice chronically infected with MCMV were incubated for 4 days with clone medium (RPMI with 10% FBS, 10^{-5} M 2-ME, and 10% rat con A supernatant) and the M45 peptide at a concentration of 10^{-10} M. Recombinant human IL-2 (100 U) was added on day 4 and the medium was replaced with fresh clone medium. The cells were used 7 days after one round of stimulation with the M45 peptide.

Viruses

MCMV Smith was purchased from American Type Culture Collection (1399-VR). ΔMC96.24 (Δm152) and rMC96.27 (wild-type rescuant) (10), ΔMS94.5 (lacking open reading frames *m151–165*) (9), BAC-derived wild-type virus MW97.01 (26), and Δm152-MW99.05 (3) have been described.

Mice

Six-week-old female C57BL/6 mice were purchased from Simonsen Laboratories (Gilroy, CA). $K^{b-/-}$ mice, the gift of F. Lemmonier (Institute Pasteur, Paris, France), were maintained in our animal facilities at Oregon Health and Science University (Portland, OR) and were used in experiments no earlier than 6 wk postbirth.

CTL assays

Either IFN-γ-pretreated, B6 mouse embryo fibroblasts (MEF) or untreated JAWS II cells (CRL-11904; American Type Culture Collection) were used as virus-infected targets. The addition of IFN-γ to JAWS II cells does not alter Ag presentation to MCMV-specific CD8s and therefore was not added to the cultures (M. C. Gold, unpublished data). Cells were infected with 50 PFU (except when noted) of virus for 16 h in the presence of 300 μg/ml phosphonoacetic acid (PAA; Sigma-Aldrich, St. Louis, MO) to prevent L gene expression. IE only or IE and E gene expression were enhanced as previously described (15). Briefly, to selectively enhance IE gene expression, B6 targets were infected with MCMV in the presence of cycloheximide (50 μg/ml; Sigma-Aldrich) followed by actinomycin D (5 μg/ml; Sigma-Aldrich). For IE and E gene expression, cells were infected in the presence of cycloheximide alone followed by a 3-h incubation without drugs. For peptide screening, 10^4 ^{51}Cr-labeled RMA-S target cells were incubated with peptide at the concentrations indicated and plated with CTL clones at an E:T ratio of 15:1. After 5 h, the amount of radioactivity (cpm) in the supernatant was counted using a TopCount (Packard Instrument, Meriden, CT). The percentage of specific lysis was determined from the following equation: ((cpm experimental release − cpm medium release)/(cpm total release − cpm medium release)) × 100.

ICS assay

Splenocytes were isolated from MCMV-infected mice. APC (JAWS II cells) were infected with MCMV (Δm152-MW99.05) at an MOI of 100 for 16 h in the presence of PAA. Effector splenocytes were incubated with peptide at various concentrations, or at a ratio of 1:1 with infected or uninfected JAWS II cells for 5 h in the presence of brefeldin A (GolgiPlug; BD PharMingen, Franklin Lakes, NJ). Cells were washed, incubated with FcBlock (BD PharMingen), and surface stained with an Ab to the CD8α chain (BD PharMingen). Cells were then fixed and permeabilized using BD PharMingen's Cytofix/Cytoperm kit before staining with an Ab to IFN-γ (BD PharMingen). $CD8^+$ T cells were analyzed by flow cytometry using FACS in conjunction with CellQuest software (BD PharMingen). All further analyses were performed using FlowJo software (Treestar, San Carlos, CA).

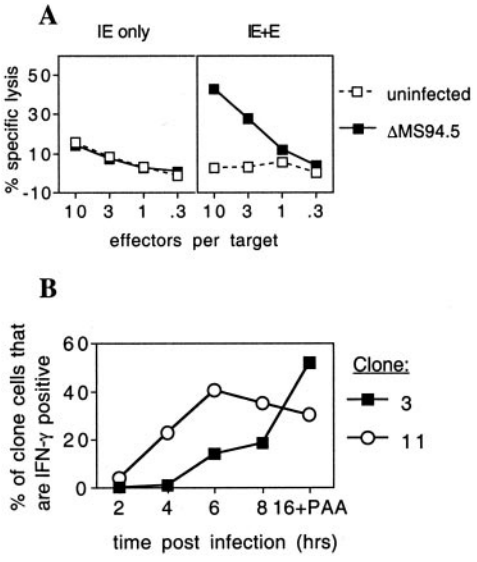

FIGURE 1. *A*, Clone 3 recognizes an E MCMV Ag. B6 MEF were infected with ΔMS94.5 (lacks *m152*) under conditions that restrict MCMV gene expression to IE genes only (*left panel*) or to IE and E gene expression (*right panel*). *B*, Clone 3 recognizes an E Ag expressed later in E gene expression. B6 MEF were infected with ΔMS94.5 at an MOI of 50 for the times indicated in the absence of PAA, or for 16 h in the presence of PAA. CTL clones were then incubated with these cells for 5 h in the presence of brefeldin A (inhibiting further Ag expression) and analyzed by ICS. Ag presentation to clone 3 was first detected at 6 h postinfection; in contrast, clone 11, specific for a K^b-restricted E Ag (data not shown), detected Ag at 4 h postinfection.

RMA-S stabilization assay

HGIRNASFI and KHGIRNASFI peptides were titrated from 10^{-6} to 10^{-13} M in RPMI supplemented with 10% FBS, added to RMA-S cells, and incubated overnight at 23°C. The next day, the cells were washed four times in PBS (pH 7.4) at 23°C to remove excess peptide and incubated at 37°C for 4 h. Only peptide-loaded class I MHC is stable at the cell surface at this temperature (27). Cells were washed, then surface stained using the D^b-specific mAb B22.249 (American Type Culture Collection) followed by FITC-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and analyzed by flow cytometry as above.

Results

D^b-restricted CTL clones isolated from a mouse infected with virus lacking m152 *recognize an E gene-encoded Ag*

A panel of CTL clones was isolated from B6 mice infected with various MCMV strains (3) and was used to screen an expression library of random MCMV DNA fragments, as described in *Materials and Methods*. Two CTL clones (clones 3 and 55) were isolated from mice infected with the MCMV mutant ΔMS94.5, which lacks 14 genes including *m152* (9). Clones 3 and 55 have been previously shown to be D^b restricted (3). Drug blockade experiments either to limit herpesvirus gene expression to IE genes or to allow E gene expression while blocking L gene expression indicated that clone 3 was specific for an E Ag (Fig. 1*A*). E gene expression occurs in waves, with *m152* expression occurring in the earliest wave, 2 h postinfection (5, 20). We predicted that Ags expressed after *m152* is expressed would be most susceptible to the effects of *m152*. To determine when in the infectious cycle the Ag recognized by clone 3 is expressed, we asked when it could be presented if *m152* were absent. To this end, we infected cells with virus lacking *m152* and used intracellular cytokine staining (ICS) to determine how many hours of infection were required for Ag recognition by clone 3. The presence of brefeldin A in the ICS assay precludes further export of MHC class I in the infected cells and thus restricts the MHC-peptide complexes detected to those already at the cell surface at the indicated time postinfection. Fig. 1*B* shows that clone 3 first detected Ag 6 h postinfection, and that recognition increased thereafter (Fig. 1*B*). This is well after *m152* expression, which commences 2 h postinfection (5, 20). To confirm that the assay was capable of detecting Ag expression earlier in the infectious cycle we included for comparison another MCMV-specific CTL clone. Fig. 1*B* shows that clone 11 detected Ag by 4 h postinfection and showed quite different kinetics. Therefore, clone 3 detects an Ag expressed in the infectious cycle after *m152* is active. We concluded that the Ag recognized by clone 3 would be a useful indicator with which to investigate the effect of the immunomodulatory genes on immunodominance.

D^b-restricted CTL clones recognize aa 985–93 of M45

From the MCMV genomic expression library, we identified a plasmid that both CTL clones 3 and 55 recognized. This plasmid encoded a small fragment of the *M45* gene (28). Based on the published MCMV sequence (28) and the corrected addition of a cytosine at nucleotide position 61918 of the MCMV genome (29), *M45* encodes a 1174-aa protein that is homologous to human CMV *UL45* and to the large subunit of the class Ia murine ribonucleotide reductase gene (28).

From the translated sequence of the expressed *M45* fragment, we predicted four potential D^b-binding peptides that were synthesized and used to sensitize RMA-S cells for lysis by clones 3 and 55 in a ^{51}Cr release assay. Fig. 2 shows that both the 9-mer HGIRNASFI (aa 985–993) and the 10-mer KHGIRNASFI (aa 984–993) were equally able to sensitize targets for killing by clones 3 and 55. Both peptides sensitized targets down to 10^{-12} M. As a measure of the relative affinity of HGIRNASFI and KHGIRNASFI, we compared their ability to stabilize cell surface D^b in the TAP-deficient cell line, RMA-S (27). As shown in Fig. 3, the 9-mer and the 10-mer were equally effective at stabilizing cell surface D^b. It is likely that the 9-mer represents the minimal epitope with the histidine residue occupying the A pocket of D^b (30).

Detection of $M45_{985–93}$-specific CD8s directly ex vivo

We next determined whether we could detect HGIRNASFI-specific CD8 T cells directly ex vivo. Because of the low frequency of

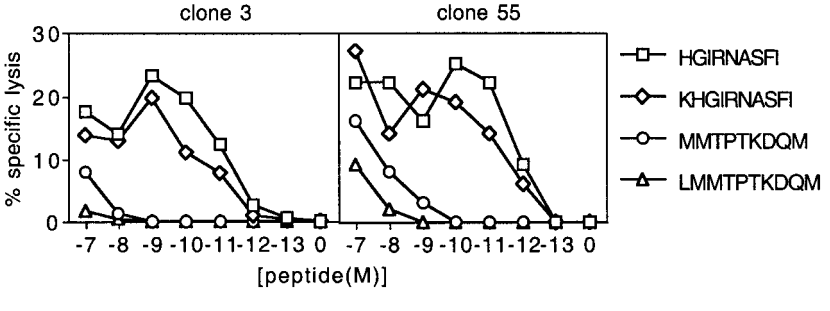

FIGURE 2. Clones 3 and 55 recognize HGIRNASFI and KHGIRNASFI peptides. Clones 3 and 55 were used as effectors in a ^{51}Cr release assay using RMA-S target cells loaded with four peptides titrated from 10^{-7} to 10^{-13} M.

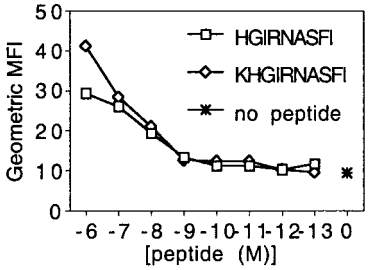

FIGURE 3. HGIRNASFI and KGHIRNASFI are equally able to stabilize the D^b class I molecule. Peptide stabilization of cell surface D^b on RMA-S cells was analyzed by flow cytometry as described in *Materials and Methods*. Data are representative of two separate experiments.

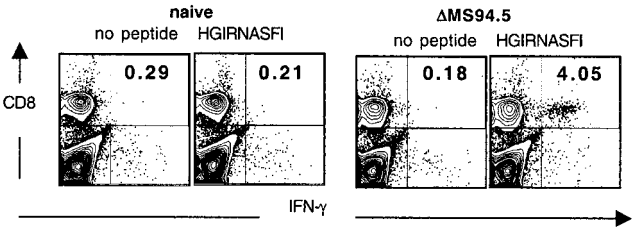

FIGURE 5. Naive mice do not respond to HGIRNASFI. Splenocytes from B6 mice infected with ΔMS94.5 for 12 wk or naive mice were tested by ICS for their response to peptide (10^{-7} M). The number in the *upper right quadrant* represents the percentage of CD8s that produced IFN-γ. Two mice were tested under each condition; one representative analysis is shown. Data are representative of three experiments.

CD8s responding to previously identified E Ags, we optimized our chances of detecting (K)HGIRNASFI-specific CD8 T cells in vivo by looking at the peak of the response in mice whose only class I molecule is D^b. $K^{b-/-}$ mice were infected with the MCMV mutant ΔMS94.5 (which lacks 14 genes, including *m152*). The ICS assay was used to quantify the frequency of M45-specific $CD8^+$ spleen cells from acutely infected mice. Over 20% of all $CD8^+$ splenocytes from $K^{b-/-}$ mice infected for 6 days with ΔMS94.5 were (K)HGIRNASFI-specific (Fig. 4). We were encouraged by the strength of this response to look at the CD8 T cell response in chronically infected B6 mice. Fig. 4 shows the result from a representative mouse infected with MCMV-Δm152 for 1.5 years, in which 2.5% of $CD8^+$ splenocytes recognized the (K)HGIRNASFI peptides (Fig. 4). Finally, no response to HGIRNASFI was detected in a naive B6 mouse, while in the same assay 4% of CD8s from a mouse infected with ΔMS94.5 for 12 wk made IFN-γ in response to HGIRNASFI (Fig. 5). In another experiment with four B6 mice chronically infected with viruses lacking *m152*, between 1 and 4% of CD8s were specific for HGIRNASFI, whereas <0.3% of CD8s were positive in two naive mice (data not shown). Other experiments suggest that the difference in the magnitude of the HGIRNASFI-specific responses seen in Fig. 4 is most likely to be due to differences between the acute and chronic phase of the CD8 response rather than a difference between $K^{b-/-}$ mice and B6 mice. Future studies are planned to determine whether the HGIRNASFI-specific response changes in its relative proportion to the total MCMV-specific response over time in mice chronically infected with MCMV.

m152 *ablates recognition of infected target cells by M45-specific CTL*

The effects of the immunomodulatory gene *m152* on M45 presentation in vitro are profound. Fig. 6*A* shows that clones 3 and 55 were unable to kill fibroblasts infected with three different wild-type MCMV strains. However, this defect was reversed if *m152* was absent: clones 3 and 55 were able to kill fibroblasts infected with three different MCMV mutants lacking *m152*. *m152* was equally effective at inhibiting presentation of M45 in MEFs and the dendritic cell (DC) line, JAWS II, that was used as the APC in the ICS assays. Fig. 6*B* shows a CTL assay using a polyclonal M45-specific CTL line: wild-type infected targets were not recognized, whereas Δm152-infected targets were readily lysed. We also tested this line for IFN-γ release by ICS, using both MEFs and JAWS II cells as APCs. Wild-type MCMV-infected MEFs and JAWS II cells failed to stimulate IFN-γ secretion, whereas both cell types infected with MCMV-Δm152 did stimulate IFN-γ secretion (data not shown). We conclude that *m152* effectively inhibits presentation of HGIRNASFI in both MEFs and a DC line.

m152 *does not affect the immunodominance of M45 in vivo*

Because *m152* appeared able to completely prevent presentation of M45, we hypothesized that mice infected with a wild-type ($m152^+$) MCMV would not develop an M45-specific CTL response. To test this prediction, we infected $K^{b-/-}$ mice with wild-type MCMV (Smith), ΔMS94.5, ΔMC96.24 (Δm152), and the rescued virus rMC96.27 (ΔMC96.24 with *m152* restored). Fig. 7 shows that, surprisingly, the percentage of CD8s recognizing M45 was very similar in each infection: between 12 and 13% of $CD8^+$ splenocytes on day 8 postinfection were specific for M45.

Although mice infected with wild-type virus made a response to M45, we reasoned that *m152* might nevertheless affect the immunodominance of M45 within the hierarchy of the total CD8 response to MCMV. We were unable to test this hypothesis by comparing the M45 response to other specific peptides because no other $H\text{-}2^b$-restricted epitopes have yet been identified. Instead, we

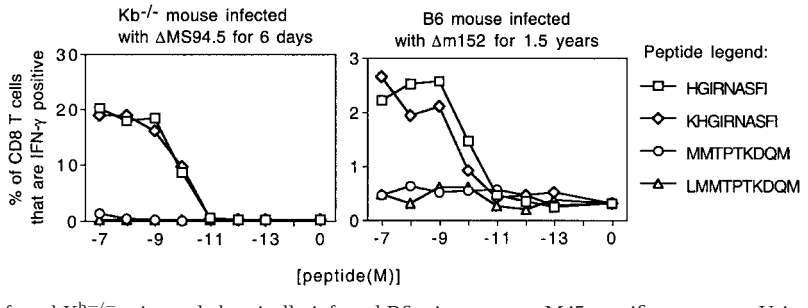

FIGURE 4. Both acutely infected $K^{b-/-}$ mice and chronically infected B6 mice generate M45-specific responses. Using the ICS assay, $CD8^+$ splenocytes were tested for their ability to respond to M45 peptides. Peptides were titrated from 10^{-7} to 10^{-13} M and were incubated with ex vivo splenocytes in the presence of brefeldin A for 5 h. CD8s were analyzed by ICS. *Left panel*, CD8s were from a $K^{b-/-}$ mouse infected with ΔMS94.5 for 6 days. *Right panel*, CD8s were from a B6 mouse infected with Δm152-MW99.05 for 1.5 years. In two similar experiments using at least two mice per group, similar numbers of CD8s responded to 10^{-8} M peptide.

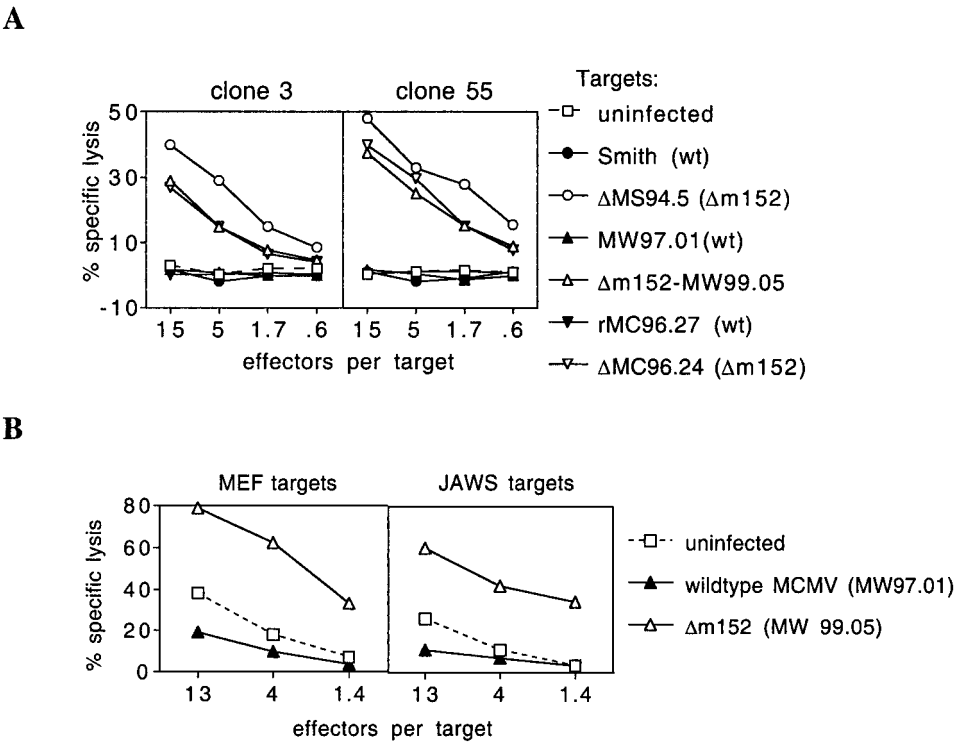

FIGURE 6. *A, m152* prevents Ag recognition by clones 3 and 55. Clones 3 and 55 were tested in a CTL assay with three different wild-type MCMV strains or with three mutants lacking *m152*; only the mutants were recognized. These results are typical of multiple experiments. *B, m152* interferes with the ability of an M45-driven CTL line to kill both MEFs and JAWS II target cells infected with MCMV. M45-specific effectors were splenocytes from $K^{b-/-}$ mice infected with wild-type MCMV stimulated in vitro with 10^{-10} M HGIRNASFI peptide for 1 wk. Target cells were either B6 MEFs or JAWS II cells infected as indicated. The reduced killing on wild-type-infected targets compared with the killing seen on uninfected targets is frequently seen using polyclonal CTL and may reflect NK activity. A duplicate of this experiment showed similar results.

calculated the ratio of the M45-specific response to the response we could detect to the whole virus, which was assessed using virus-infected APCs in the ICS assay. This time we used B6 mice, where D^b-HGIRNASFI also competes with K^b-restricted responses for immunodominance. Five mice were infected with the MCMV mutant Δm152 (ΔMC96.24) or the rescuant (rMC96.27) MCMV strains for 8 days. Fig. 8 shows that CD8 T cell responses to MCMV (Δm152)-infected DCs ranged from 3 to 11%. No remarkable differences were seen in the CD8 T cell responses to virus-infected APC after MCMV infection with or without *m152*. This indicated that *m152* did not affect the total numbers of MCMV-specific CD8 T cells that were generated in vivo. This assay also allowed a rough assessment of the immunodominance of M45 within the total MCMV-specific CD8 response. While the HGIRNASFI peptide stimulation probably detects all CD8s of this specificity, it is likely that simulation with virus-infected DCs underestimates the total number of MCMV-specific CD8s. Therefore, we conclude that, while a substantial number of CD8s recognized HGIRNASFI, other specificities exist. Finally, we compared the HGIRNASFI-specific response to the detectable response to whole virus after infection with MCMV with or without *m152*. Although there was mouse to mouse variation, the ratio of the peptide-specific response to the total MCMV-specific response did not differ significantly ($p = 0.39$). The ratio of the M45-specific CD8 T cell response to the total MCMV-specific CD8 response averaged among five mice (expressed as a percentage) was 62.1% after MCMVΔm152 infection vs 72.8% after infection with the rescuant (Fig. 8 and Table I). These data again indicate that *m152* did not alter the immunodominance of the M45 Ag.

Discussion

Identification of an immunodominant MCMV CTL Ag in B6 mice

In this paper we describe the use of CTL clones and a genomic fragment library to identify an immunodominant epitope in MCMV. Identification of CTL epitopes in large viruses remains a difficult procedure, and several points in the method described here are worthy of note. To initially isolate the CTL clones we screened

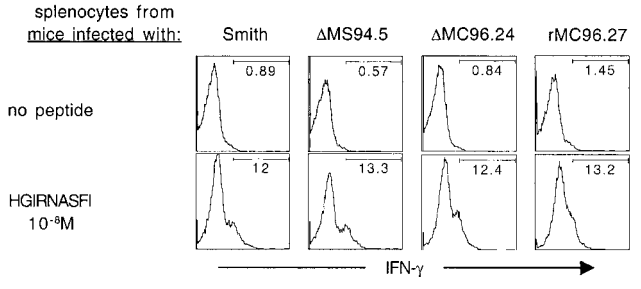

FIGURE 7. *m152* does not affect the M45-specific response in acutely infected $K^{b-/-}$ mice. Shown is quantification by ICS assay of HGIRNASFI-specific $CD8^+$ splenocytes from $K^{b-/-}$ mice infected for 8 days with wild-type (Smith), ΔMS94.5, ΔMC96.24 (Δm152), and rMC96.27 (revertant) viruses. The number in the corner indicates the percentage of CD8 T cells specific for M45. Similar results were seen in two separate experiments with two mice per group.

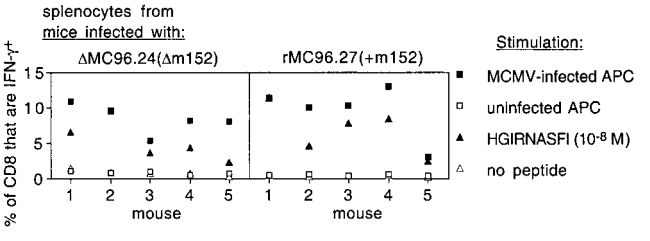

FIGURE 8. *m152* does not affect the immunodominance of M45 in acutely infected B6 mice. HGIRNASFI-specific and total MCMV-specific responses were compared by ICS assay. Splenocytes from B6 mice infected for 8 days with Δm152 (ΔMC96.24) or revertant (rMC96.27) were incubated for 6 h either with 10^{-8} M HGIRNASFI peptide or with Δm152-infected JAWS II cells used as APCs. Data are representative of three experiments with a minimum of two mice per group.

Table I. *Calculated ratio of the HGIRNASFI-specific response to the response to virus-infected APCs*[a]

Mouse	MCMV Infection (%)	
	ΔMC96.24 (Δm152)	rMC96.27 (wild-type)
1	60.8	100
2	99.2	45.6
3	68.8	76.1
4	53.1	64.3
5	28.6	77.8
Average ± SD	62.1 ± 25.6	72.8 ± 19.9

[a] The frequency of M45-specific CD8s expressed as a percentage of the frequency of total MCMV-specific responses as assessed using virus-infected APCs using results from Fig. 8.

clones for their ability to kill targets infected with a virus (ΔMS94.5) that lacks the key immune evasion gene, *m152*. The CTL clones used in this paper were generated from Δm152-infected mice, but we have also generated clones from wild-type-infected mice (3); CTL clones from both types of infection were able to lyse ΔMS94.5-infected targets but not wild-type-infected targets. Thus, the use of a virus lacking immune evasion genes to screen CTL clones was critical for isolation of these clones. M45 is immunodominant in wild-type infection, but clones of this specificity would not have been identified had we used wild-type virus in our initial screen. The use of viruses lacking immune evasion genes for screening clones may also be useful in identifying new CTL epitopes in other viruses such as human CMV, HSV, and Kaposi's sarcoma-associated herpesvirus.

The second feature of the strategy described here was the use of a viral genomic fragment library. This strategy has previously been applied successfully to identify epitopes in *Chlamydia* and HSV (S. P. Fling, unpublished results). If a pathogen's genomic DNA can be obtained independently of the host genome this approach can be useful, because it eliminates contaminating host transcripts that would be expressed in a cDNA library and which would increase the clonal complexity of the library. Another advantage of expression cloning in general is the potential to identify both dominant and subdominant epitopes. We do not yet know whether genomic libraries constructed by restriction digest express all possible epitopes. Clearly, if a restriction site coincides with the portion of DNA encoding an epitope, representation may be limited. However, this problem is abrogated to some extent by partial restriction digests of input DNA.

The M45 epitope is clearly recognized by a substantial fraction of MCMV-specific CD8s in B6 mice. Identification of other epitopes is necessary before we can determine whether it is the most immunodominant epitope in this strain. The only other immunodominant MCMV epitopes identified so far are for the H-2^d haplotype: epitopes from pp89 (L^d-restricted) (12, 13) and m164 (D^d-restricted) (23) have been described. Reddehase and colleagues (21, 23) injected MCMV into the footpad, measured the CTL response using an ELISPOT assay, and determined that ~0.7% of CD8s in the spleens of chronically infected BALB/c mice were specific for each of these epitopes. These authors used the number of CD8s that made IFN-γ in response to anti-CD3 (~2%) to identify previously activated CD8s and argued that this number approximated the total MCMV-specific memory population. Based on this assumption they concluded that most MCMV-specific CD8s in BALB/c mice are specific for either pp89 or m164. Very recently, Ye et al. (14) used a protocol similar to the one we used in the current paper and determined that pp89-specific CD8s made up close to 3% of CD8s 1 mo after infection. We measured a similar response to pp89 (4% of CD8s) in one experiment in BALB/c mice (M. C. Gold, unpublished observation). We think it more likely that differences in infectious route and method of detecting the response, rather than between BALB/c and B6 mice per se, will be found to account for these differences in the measured size of the response. It will be interesting to perform side by side comparisons using the same methods to determine the relative size of the overall MCMV-specific response in both mouse strains, as well as the proportion of that response that can be attributed to the currently identified epitopes.

m152 *affects presentation of M45 without affecting its immunodominance*

The hypothesis that *m152* would affect the hierarchy of immunodominant CD8 epitopes is based on the fact that *m152* severely impairs Ag processing and presentation in fibroblasts. However, our work demonstrates that the CD8 T cell response to M45 is not affected by the presence of *m152* in vivo even though its presentation is severely affected by *m152* in vitro. We can think of two possible explanations for this surprising result. First, Ag presentation in some important cell types in vivo, such as macrophages or DCs, may not be affected by *m152* in the same way that fibroblasts are affected in vitro. It has been reported that macrophages can overcome the effect of immune evasion genes and effectively present the IE pp89 Ag (31). However, our experience is that M45-specific CTL clones are unable to kill wild-type virus-infected macrophages, even though they readily kill Δm152-infected macrophages (D. LoPicollo, M. Gold, D. Kavangh, M. Wagner, U. Koszinowski, and A. Hill, manuscript in preparation). Similarly, we show in this paper that *m152* effectively prevents Ag presentation in a DC line. Thus, while it remains possible that presentation of M45 by an infected cell in vivo is not affected by *m152*, there is currently no evidence to support that hypothesis.

A second explanation for the lack of effect of *m152* on the immunodominance of M45 could be that cross-presentation is the principle mechanism for priming the CD8 T cell population to M45 in vivo. Cross-presentation is the process by which DCs take up Ag from infected cells and present it on newly synthesized class I molecules. Presumably *m152*, a glycoprotein that acts in the ER, only affects Ag presentation in infected cells and does not affect cross-presentation. Cross-presentation is believed to be an important mechanism by which CD8 T cells are primed in vivo (32, 33), and it is thus perhaps not surprising that M45-specific CD8 T cell responses would be elicited in wild-type MCMV-infected mice. Nevertheless, if cross-presentation is the main mechanism used for priming the CTL response, this could potentially be problematic for the immune response. If the Ags presented by cross-priming are different from those presented directly by virus-infected cells, then the immune response may be fooled into making a CTL response that is ineffective in clearing virus-infected cells. Indeed, we had assumed the CD8 T cell response would focus on Ags that are efficiently presented by virus-infected cells in vivo. In an attempt to reduce in vitro artifact, we assessed the efficacy of immune evasion genes using virus infections (rather than transfections) of primary untransformed murine fibroblasts, as well as a DC line. In these assays *m152* prevented recognition of the M45-encoded epitope. However, we do not yet know how effective M45-specific CD8s are in vivo against wild-type virus or, indeed, for which infected cell types in vivo CD8 control is most important. However, we do note that MCMV is effectively controlled by the immune response and that CD8 T cells play an important role in this control. Future studies will address these issues.

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