

Protection against Lethal Cytomegalovirus Infection by a Recombinant Vaccine Containing a Single Nonameric T-Cell Epitope

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The regulatory immediate-early (IE) protein pp89 of murine cytomegalovirus induces CD8⁺ T lymphocytes that protect against lethal murine cytomegalovirus infection. The IE1 epitope is the only epitope of pp89 that is recognized by BALB/c cytolytic T lymphocytes (CTL). Using synthetic peptides, the optimal and minimal antigenic sequences of the IE1 epitope have been defined. To evaluate the predictive value of data obtained with synthetic peptides, recombinant vaccines encoding this single T-cell epitope were constructed using as a vector the hepatitis B virus core antigen encoded in recombinant vaccinia virus. In infected cells expressing the chimeric proteins, only IE1 epitope sequences that were recognized as synthetic peptides at concentrations lower than 10⁻⁶ M were presented to CTL. Vaccination of mice with the recombinant vaccinia virus that encoded a chimeric protein carrying the optimal 9-amino-acid IE1 epitope sequence elicited CD8⁺ T lymphocytes with antiviral activity and, furthermore, protected against lethal disease. The results thus show for the first time that recombinant vaccines containing a single foreign nonameric CTL epitope can induce T-lymphocyte-mediated protective immunity.

Antigenic sequences recognized by cytolytic T lymphocytes (CTL) in viral protein antigens can be identified by using synthetic peptides (23; reviewed in reference 22). Synthetic peptides also permit definition of the minimal and optimal sequences that bind to major histocompatibility complex class I proteins and are recognized by the antigen-specific T-cell receptor. However, the set of peptides naturally produced in virus-infected cells by processing of viral proteins may differ substantially from the optimal sequences defined with synthetic peptides. Presentation to CTL has been shown recently for epitopes of 13 to 22 amino acids (aa) expressed from chimeric proteins synthesized in transfected and infected cells (5, 10, 26). However, chimeric proteins have failed to induce a T-lymphocyte response in vivo (13). Knowledge of the features that affect antigenicity and immunogenicity of epitopes expressed from endogenously synthesized proteins is crucial for design of vaccines containing CTL epitopes.

A need for separate epitopes recognized by helper T lymphocytes, the nature of the carrier protein, and the length and antigenic potency of the inserted antigenic sequence itself may affect the immunogenic potential of recombinant vaccines. We have addressed these issues in a herpesvirus model, murine cytomegalovirus (MCMV) infection, in which a protective antiviral immune response mediated by CD8⁺ T lymphocytes is induced by the nuclear viral phosphoprotein pp89 (11, 17, 18, 20, 25). An epitope of pp89 that is recognized by CTL clone IE1 (15) has been identified and named the IE1 epitope (7). Using synthetic peptides, the optimal antigenic sequence 168-YPHEMPTNL-176 for CTL clone IE1 has been defined. Shorter peptides such as PHEMPTN and the core HFMPT are also recognized, but their antigenic potencies are 10³- and 10⁶-fold lower, respectively (19).

To examine the predictive value of data obtained with synthetic peptides for the construction of recombinant T-cell

epitope vaccines, we inserted DNA sequences encoding IE1 epitope sequences of various lengths and antigenic potencies into different genetic environments. The capacity of the resulting chimeric proteins to elicit an effective in vivo antiviral T-lymphocyte response was studied. Protection against lethal virus challenge was successfully achieved with a chimeric protein containing a single, 9-aa T-cell epitope.

MATERIALS AND METHODS

Mice. BALB/c mice (*H-2^d* haplotype) were bred in our colony under specific-pathogen-free conditions.

Cells. Third-passage mouse embryo fibroblasts were used for infection with MCMV to produce target cells and for determination of virus titers. KD2SV, a simian virus 40-transformed kidney fibroblast line established from B10.D2 mice (*H-2^d* haplotype) (14), was used for infection with recombinant vaccinia viruses to produce target cells.

Viruses. Purified, tissue-culture-grown MCMV of strain Smith (VR-194; American Type Culture Collection, Rockville, Md.) was used for production of target cells, for generation of CTL, and for infection of mice in adoptive transfer experiments. Challenge infection of immunocompetent vaccinated mice was performed with virulent MCMV isolated from the salivary glands of infected mice (11).

Recombinant vaccinia viruses. The recombinant vaccinia virus MCMV-*ie1*-VAC encodes pp89 (25). The recombinant MCMV-*ie1*(Δ F)-VAC encodes a deletion mutant of pp89 that lacks amino acids 136 to 249 and therefore does not contain any epitope recognized by BALB/c CTL (7).

(i) Deletion of IE1 epitope pentameric core: Δ 5-mer construct. The nucleotide sequence encoding the IE1 epitope core (170-HFMPT-174) was deleted by site-directed mutagenesis in M13mp19 using the oligonucleotide 5'GCC CTA GAT TTG GGT ACA TG3' and following the procedure described by Zoller and Smith (27) as modified by Volkmer et al. (25). Oligonucleotides were synthesized in an Applied Biosystems oligonucleotide synthesizer (model 381A). Mutagenesis was controlled by DNA sequencing. The mutated

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pp89 sequence was cloned downstream of the vaccinia virus P7.5 early/late promoter into the vaccinia virus recombination vector pCS43 (1). Recombinant vaccinia viruses were produced as described earlier (25).

(ii) **Integration of IE1 epitope sequences in deletion mutant ΔF .** To reintegrate IE1 epitope sequences at a different position in pp89, we chose deletion mutant ΔF , which lacks the IE1 epitope. A unique *Bgl*III site in the 3' end of the ΔF reading frame, at codon 553 (7, 12), was used as the insertion site for synthetic oligonucleotides. The oligonucleotides contained IE1 epitope sequences and were flanked by a *Bam*HI site at their 5' end and by an *Eco*RI and a *Bgl*III site at their 3' end. The oligonucleotide encoding the 7-mer sequence was integrated for a second time in the reverse orientation. All constructs were sequenced and then recombined into vaccinia virus.

(iii) **Integration of pp89 IE1 epitope sequences into HBcAg.** An *Ava*I site located 5 codons upstream of the termination codon of the hepatitis B virus core antigen (HBcAg) gene of hepatitis B virus type 2 (9) was used for the insertion of synthetic oligonucleotides encoding IE1 epitope sequences. Sequence analysis was performed before recombination into vaccinia virus.

Cytolytic assays. (i) Target cells. Enhanced and selective expression of MCMV immediate-early gene products including pp89 was achieved as described previously (16). For expression of foreign protein antigens by recombinant vaccinia viruses, KD2SV cells were infected with 5 to 20 PFU per cell for 16 h. Target cells were trypsinized and labeled for 90 min with $\text{Na}_2^{51}\text{CrO}_4$, and a standard 3-h cytolytic assay was performed with 1,000 target cells and graded numbers of cytolytic effector cells in twofold or threefold dilution steps. Data represent the mean percentage of specific lysis from three replicate cultures. For preparing target cells with exogenously added peptides, 10^5 cells were incubated with peptides at different concentrations in a volume of 50 μl for 1 h at room temperature after the ^{51}Cr labeling, and excess unbound peptide was washed off before effector cells were added.

(ii) **Cytolytic effector cells.** pp89-specific polyclonal CTL were generated by in vitro restimulation with purified MCMV of spleen cells from mice that had been intravenously infected with 10^8 PFU of recombinant MCMV-*ie1*-VAC 3 weeks before (7).

Western blot (immunoblot) analysis. KD2SV cells were infected with 5 to 20 PFU of the recombinant vaccinia viruses per cell for 16 h, lysed in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, separated by electrophoresis, and blotted onto nitrocellulose membranes. For detection of recombinant pp89 proteins, blots were incubated with a rabbit antiserum raised against amino acid residues 34 to 53 of pp89 (7). For detection of recombinant HBcAg proteins, a human patient serum sample reactive with HBcAg was used.

Adoptive transfer. Donor mice used as source of CD8⁺ T lymphocytes were immunized with MCMV (2×10^5 PFU intraperitoneally) or with recombinant vaccinia viruses (1×10^8 PFU intravenously) at least 3 weeks earlier. Splenic CD8⁺ T lymphocytes were enriched by passage through a nylon wool column and subsequent treatment with antibody to CD4 and complement (20), and their in vivo antiviral activity was tested by adoptive transfer into sublethally irradiated (5.5 Gy, corresponding to a 50% lethal dose [LD_{50}] over an observation period of 30 days) and MCMV-infected (2×10^5 PFU in the footpad) 9-week-old recipient BALB/c mice as described previously (20). Twelve days after trans-

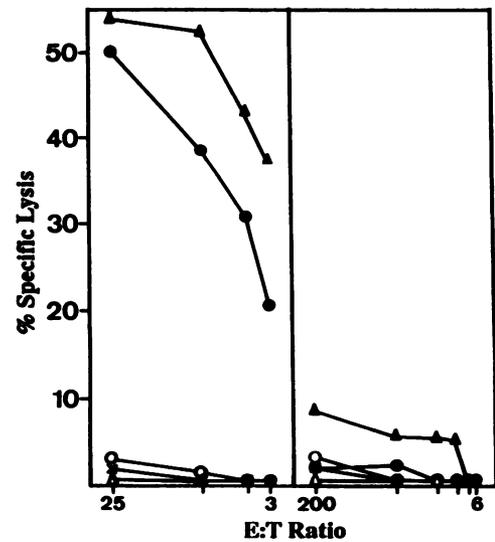


FIG. 1. IE1 epitope sequences are essential for the recognition of pp89 by BALB/c CTL. Spleen cells from mice primed with the recombinant vaccinia virus MCMV-*ie1*-VAC, encoding pp89 (left panel), or with the recombinant $\Delta 5$ -mer, encoding a deletion mutant of pp89 that lacks the IE1 epitope pentameric core (right panel), were restimulated in vitro with MCMV and tested in a cytolytic assay for recognition of MCMV-infected target cells expressing pp89 (▲) and of target cells infected with MCMV-*ie1*-VAC (●) or $\Delta 5$ -mer (○). Cells infected with wild-type vaccinia virus (×) and uninfected target cells (Δ) provided the negative controls. E:T Ratio, effector-to-target cell ratio.

fer, MCMV titers in spleens of individual recipient mice were determined.

Protection against lethal challenge infection. Mice were vaccinated i.v. with 10^8 PFU of recombinant vaccinia viruses, and 3 weeks later, they were challenged i.p. with 3 LD_{50} s of virulent salivary gland-derived MCMV (11).

RESULTS

IE1 epitope sequences are essential for recognition of pp89 by BALB/c CTL. To test whether single epitopes recognized by CTL can replace the whole protein for induction of protective immunity in vivo, knowledge of the number of epitopes present in the protein is important. Previous results showed that the region of pp89 between amino acid residues 136 and 249 was required for recognition of the protein by CTL (7). Although the IE1 epitope presented by L^d has been positively identified within this region (6, 7), it was not excluded that other epitopes exist. To detect epitopes besides IE1, a deletion mutant of pp89, named $\Delta 5$ -mer, was constructed and recombined into vaccinia virus. This recombinant lacked just the pentameric core HEMPT of the IE1 epitope. When it was used to infect target cells expressing all $H-2^d$ class I molecules, the mutant protein was not recognized by polyclonal pp89-specific CTL from BALB/c mice primed with the recombinant MCMV-*ie1*-VAC encoding authentic pp89 (Fig. 1, left panel). In an attempt to increase the frequency of CTL specific for putative epitopes in the mutant protein, we primed mice with the $\Delta 5$ -mer recombinant. As shown in Fig. 1 (right panel), no CTL that recognized the $\Delta 5$ -mer protein or authentic pp89 could be detected. Likewise, in vitro restimulation with the $\Delta 5$ -mer recombinant of memory T lymphocytes from mice that were

latently infected with MCMV could not be achieved (data not shown). It was thus concluded that IE1 epitope sequences are essential for the recognition of pp89 by BALB/c CTL.

A 9-aa IE1 epitope sequence is efficiently presented to CTL even after shifting to a different location of pp89. The protective capacity of pp89 is based on the function of CD8⁺ T lymphocytes (11). Since IE1 is the only epitope recognized by CTL in pp89, it can be expected that it is sufficient for the induction of protective immunity. Before selecting an IE1 sequence of a given length for testing this hypothesis, it should be recalled that synthetic IE1 epitope sequences of different lengths show considerably different antigenicities for CTL clone IE1 (19). In this respect, BALB/c polyclonal pp89-specific CTL exhibit a fine specificity similar to that of CTL clone IE1, since the synthetic nonapeptide YPHFMPTNL constituted the optimal antigenic peptide, giving half-maximal recognition at a concentration as low as 10⁻¹⁰ M. The synthetic heptapeptide PHFMPTN and the pentapeptide core HFMPPT retained antigenicity, being detected at concentrations of 10⁻⁶ and 10⁻⁴ M, respectively (data not shown).

These three IE1 epitope sequences were chosen to investigate their ability to be presented when expressed from intracellularly synthesized chimeric proteins. To this end, IE1 epitope sequences of 9, 7, and 5 aa were inserted close to the C terminus of deletion mutant ΔF of pp89, which lacks the original IE1 epitope (7) (Fig. 2A). DNA constructs were confirmed by sequencing (Fig. 2B) and then recombined into vaccinia virus. Expression of the chimeric proteins was confirmed by Western blot analysis (Fig. 3A).

Antigenicity of target cells infected with the different recombinants was tested by using pp89-specific polyclonal CTL as effector cells. As shown in Fig. 4A, target cells infected with the recombinant virus encoding the ΔF/9-mer chimeric protein were recognized by CTL, thus indicating that the IE1 epitope was correctly processed and presented even when inserted into a different position of the same protein.

The 7-mer and 5-mer constructs were, however, not recognized (Fig. 4A). The same conclusion was reached when mice were immunized with the different recombinant viruses and their spleen cells were tested for the presence of pp89-specific CTL after selective in vitro restimulation with MCMV (data not shown). Additional confirmation also came from experiments in which the different recombinants were used for in vitro restimulation of pp89-specific memory CTL (data not shown). From all this evidence, it was concluded that IE1 epitope sequences shorter than 9 aa were not detectably presented from chimeric ΔF proteins, while the 9-mer was presented as efficiently as from the authentic location in pp89.

IE1 epitope is also presented from an unrelated, HBcAg-based chimeric protein. To test the protective capacity of the IE1 epitope in the absence of separate potential pp89-derived helper-T-cell epitopes, we inserted IE1 sequences of 9 and 7 aa into an unrelated protein, HBcAg. Recombinant vaccinia viruses were produced, and the sequence (Fig. 2) and expression (Fig. 3B) of the chimeric proteins were confirmed. As with the ΔF-based recombinants, when recognition by CTL of cells expressing the chimeric proteins was tested, the 9-mer construct was recognized, whereas the 7-mer was not (Fig. 4B). Thus, the same epitope presentation hierarchy was found regardless of the protein background. In addition, the fact that the nonameric IE1 epitope could be shifted not only to a different position in the same

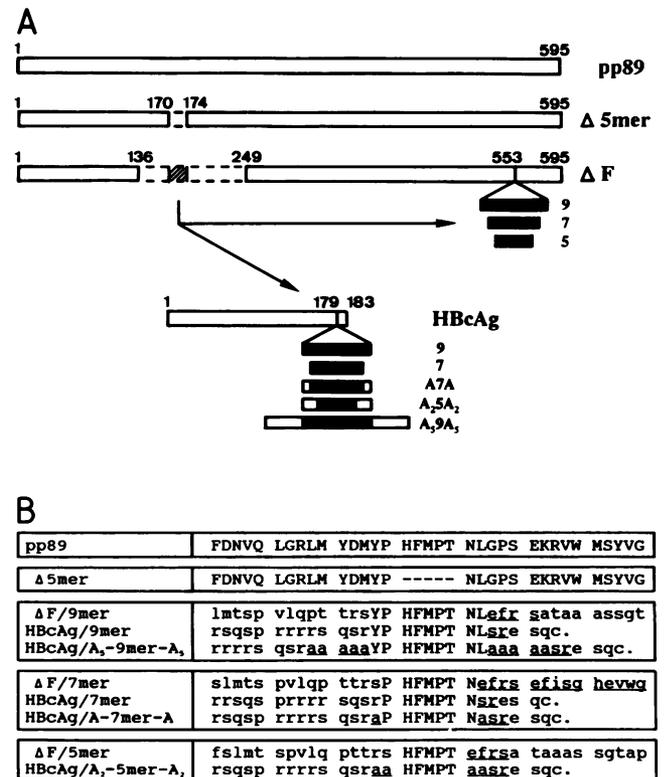


FIG. 2. Schematic representation (A) and amino acid sequence (B) of pp89 and chimeric proteins. (A) Proteins are drawn as long open boxes, and numbers above them indicate amino acid positions. IE1 epitope sequences are shown as closed boxes not drawn to the same scale as the proteins, and their length and the presence of flanking alanine residues are indicated on their right. (B) Amino acids are shown in the one-letter code. Capitals indicate native pp89 sequences. Dashes indicate deleted amino acids. Underlined lowercase letters refer to amino acids encoded by linker sequences for restriction endonucleases introduced during the construction of the chimeric genes. Lowercase indicates sequences from the vector proteins. Chimeric proteins are named, citing first the vector protein and then the length of the IE1 sequence encoded. Where alanine residues flank IE1 sequences, their number on each side of the epitope is indicated.

protein but also to an unrelated protein suggested that the requirements for its processing and presentation are not very stringent.

Correlation between antigenic potency of synthetic peptides and recognition by CTL of chimeric proteins expressing IE1 epitope sequences. The lack of recognition by CTL of the 5-mer sequence in ΔF and of the 7-mer sequence in both chimeric proteins tested could be explained either by their lower antigenicity compared with the 9-mer (19) or by their shorter length, which could bring the antigenic core into closer proximity to potentially disturbing amino acid residues derived from the vector. To discriminate between these alternatives, IE1 epitope sequences of different lengths and antigenicities were made to reach a common final length of 9 aa by flanking them with alanine residues. Alanine was chosen because it has the shortest side chain among the amino acids. The feasibility of this approach was first tested at the level of synthetic peptides. The sequence A-PHEMPTN-A has the length of the 9-mer but only the 7 aa of the IE1 epitope present in the 7-mer (PHFMPTN). When

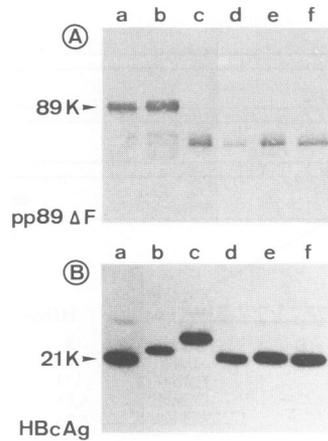


FIG. 3. Expression of pp89 and chimeric proteins containing IE1 epitope sequences in cells infected with recombinant vaccinia viruses. Western blot analysis with pp89-specific (A) or HBcAg-specific (B) antiserum of cells infected with the following recombinant viruses (see Fig. 3 for abbreviations): (A) lane a, MCMV-*ie1*-VAC, encoding pp89; lane b, $\Delta 5$ -mer; lane c, ΔF ; lane d, $\Delta F/9$ -mer; lane e, $\Delta F/7$ -mer; lane f, $\Delta F/5$ -mer; (B) lane a, HBcAg; lane b, HBcAg/9-mer; lane c, HBcAg/ A_5 -9-mer- A_5 ; lane d, HBcAg/7-mer; lane e, HBcAg/A-7-mer-A; lane f, HBcAg/ A_2 -5-mer- A_2 . K, kilodaltons.

tested as a synthetic peptide, the alanine-flanked 7-mer had a slightly higher antigenic potency than the original 7-mer. Similarly, the antigenicity of the peptide AA-HFMPT-AA was comparable to that of HFMPT (Table 1).

Once it was found that flanking alanine residues did not reduce the antigenic properties of IE1 synthetic peptides, the alanine-flanked 7-mer and 5-mer sequences were inserted into HBcAg to test whether these sequences could be presented from intracellularly synthesized proteins. Cells infected with the corresponding recombinant vaccinia vi-

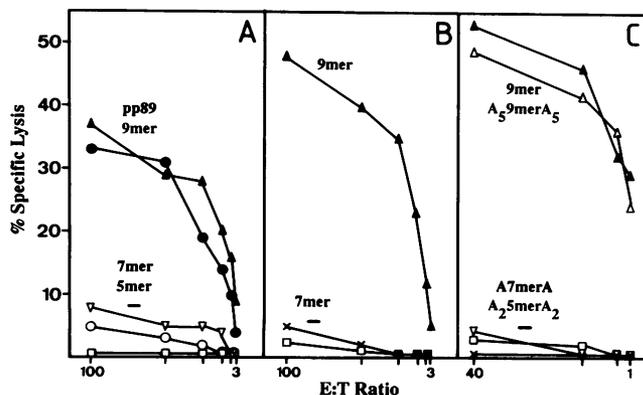


FIG. 4. The 9-aa IE1 epitope sequence is efficiently presented from intracellularly synthesized chimeric proteins. Recognition by pp89-specific CTL in a cytolytic assay of target cells infected with recombinant vaccinia viruses encoding chimeric proteins that contain IE1 epitope sequences. IE1 epitope sequences were inserted into pp89 ΔF (A) or into HBcAg (B and C). Encoded IE1 sequences are as follows: \blacktriangle , 9-mer; \square , 7-mer (A and B); ∇ , 5-mer (A); \triangle , A_5 -9-mer- A_5 ; \square , A-7-mer-A (C); ∇ , A_2 -5-mer- A_2 (C). Controls include cells infected with the recombinant viruses encoding authentic pp89 (\bullet) and the vector proteins ΔF (\circ) and HBcAg (\times). E:T Ratio, effector-to-target cell ratio.

TABLE 1. Adjustment to nonapeptide length by symmetric alanine flanking

Peptide sequence	Threshold of detection ^a	Plateau of detection ^a
YPHFMPNTL	-11 to -10	-9 to -7
PHFMPTN	-8 to -7	-4
APHFMPTNA	-9 to -8	-5 to -4
HFMPT	-5	-4 to -3
AAHFMPATAA	-6 to -5	-4

^a Recognition of peptide-incubated *L/L*^d target fibroblasts by CTL clone IE1 in a cytolytic assay. Peptide concentration is given in log molar units.

ruses were again not recognized by CTL (Fig. 4C), even though all inserts were now 9 aa long. Flanking alanine residues did not generally affect intracellular processing or presentation in a negative manner, since a chimeric protein carrying the 9-mer flanked even by five alanines in HBcAg was recognized by CTL to the same extent as was the 9-mer construct (Fig. 4C). Thus, the short length was not the determining factor for the lack of presentation of IE1 7-mer and 5-mer sequences from intracellularly synthesized chimeric proteins; rather, the lower antigenicity compared with the 9-mer was the deciding factor.

Induction of protection in vivo with the nonameric IE1 epitope sequence. Results shown so far indicate that the 9-mer IE1 epitope sequence is as antigenic when presented from chimeric proteins as the authentic pp89. We asked whether this similarity also extended to the protective capacity in vivo, that is, whether the recombinant virus encoding the 9-mer in a chimeric protein could be used as an epitope vaccine. To exclude a contribution of MCMV sequences other than the tested IE1 epitope, we used the HBcAg-based chimeric protein.

Induction of protective immunity in vivo was tested by priming mice with the different recombinant viruses followed by a challenge with a lethal dose of MCMV (Fig. 5). A high percentage of animals primed with the recombinant MCMV-*ie1*-VAC encoding the whole pp89 protein were protected

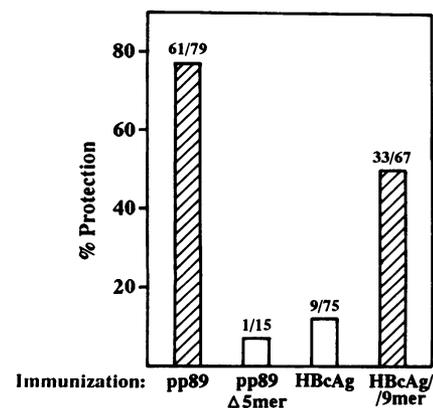


FIG. 5. Protection against lethal MCMV infection in mice immunized with pp89 IE1 epitope sequences. Mice were immunized with recombinant vaccinia viruses encoding the indicated proteins. Three weeks later they were challenged with 3 LD₅₀s of MCMV. Protection, indicated on the ordinate, is calculated as the percentage of surviving mice of the total tested. The actual number of mice in each group (survivors/tested) is indicated on top of each column. Data are compiled from nine independent experiments, three of which included the $\Delta 5$ -mer recombinant.

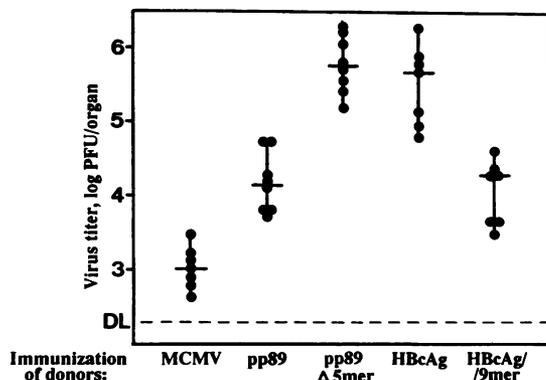


FIG. 6. Antiviral effect of CD8⁺ T lymphocytes sensitized with pp89 IE1 epitope sequences. Donor mice were immunized with MCMV or with recombinant vaccinia viruses encoding the indicated proteins. Splenic memory T lymphocytes were purified through a nylon wool column followed by depletion of CD4⁺ cells. CD8⁺ T lymphocytes thus obtained were adoptively transferred into irradiated and MCMV-infected recipient mice. Dots represent MCMV titers in the spleens of individual recipients determined at day 12 after infection. Bars represent median titer values in each group. DL, detection limit.

against death, as described previously (11). Lack of the IE1 epitope pentameric core in the Δ 5-mer recombinant resulted in the complete inability to induce protective immunity, correlating with the lack of recognition by CTL of cells infected with this recombinant virus *in vitro*. The finding that priming with HBcAg/9-mer conferred protection against lethal challenge with MCMV confirmed that induction of protection was a property of the IE1 epitope and thus showed the potential of the recombinant virus encoding the 9-mer to function as a T-cell epitope vaccine.

The efficacy of the recombinant vaccine was also tested by adoptive transfer of primed T lymphocytes into irradiated and lethally infected recipient mice (Fig. 6). This experimental system reflects more closely the clinical situation, in which infection of immunosuppressed patients with human CMV is more relevant than acute infection of immunocompetent individuals with a high dose of virus. In this model, the antiviral activity of transferred CD8⁺ T lymphocytes from donors primed with MCMV or with pp89 is reflected by a reduction of MCMV titers in different organs of infected recipient mice (11, 20) (Fig. 6). As before, removal of the IE1 pentameric core prevented induction of antiviral CD8⁺ T lymphocytes by the Δ 5-mer recombinant. Priming of the donor mice with the vaccinia virus recombinant encoding HBcAg alone did not induce antiviral T lymphocytes either. In contrast, virus titers in the spleens of the infected recipient mice were reduced after transfer of CD8⁺ T lymphocytes from mice primed with the HBcAg/9-mer recombinant to the same extent as in those mice that had received cells from donors primed with pp89. These results confirm the data from the challenge experiments and, taken together, demonstrate the induction of antiviral CD8⁺ T lymphocytes and protection against lethal infection by a recombinant vaccine containing a single nonameric T-cell epitope of the MCMV regulatory protein pp89.

DISCUSSION

CD8⁺ T lymphocytes mediate protection against lethal MCMV infection after vaccination of mice with a recombi-

nant vaccinia virus encoding a single MCMV gene product, the nonstructural nuclear phosphoprotein pp89, which is expressed in the immediate-early phase of the MCMV replication cycle (11). In the present report, we extend this finding by showing that a chimeric protein carrying a sequence of only 9 aa, which constitutes the only epitope recognized by BALB/c CTL in pp89, induces a degree of protection similar to that afforded by the entire pp89 protein.

In the lymphocytic choriomeningitis virus model, protection has been recently shown using truncated viral proteins. However, when an isolated CTL epitope was expressed in vaccinia virus, it was recognized *in vitro* by CTL but failed to induce T cells *in vivo*. A need for increased epitope expression or additional T-helper epitopes required for CTL induction was postulated (13). In our case, however, inclusion of separate sequences from pp89 or from any other MCMV protein for recognition by helper T lymphocytes was not required either for the induction or for the antiviral activity of CD8⁺ T lymphocytes. These results do not indicate any difference in the viral sequence requirements for CTL induction and recognition and thus encourage the design of multivalent vaccines that include short CTL epitopes from several viral proteins, as well as linear epitopes recognized by antibodies.

This is the first example of successful induction of protective cellular immunity using as a carrier HBcAg, a protein utilized to date only for the induction of antibodies to foreign epitopes (2). The finding that a very short sequence of pp89 can substitute for the whole protein opens the possibility of exploring antigen presentation from different carrier vectors, including those with a very limited capacity for insertion of foreign sequences. This possibility is substantiated by the finding that the IE1 epitope nonameric sequence was efficiently processed and presented from the two chimeric proteins tested.

To evaluate the antigenicity required from a candidate epitope for a recombinant vaccine, IE1 epitope sequences of different lengths and antigenicities were flanked by alanine residues to reach a common final length of 9 aa. The additional flanking alanines apparently did not affect either intracellular processing of chimeric proteins or presentation of synthetic peptides by surface class I molecules. Comparison of IE1 sequences of the same length which were inserted into the same genetic environment revealed that the IE1 epitope 7-mer (A-PHEMPTN-A) and 5-mer (AA-HEMPT-AA) sequences could not be recognized by CTL when expressed from intracellularly synthesized chimeric proteins, whereas the 9-mer (YPHEMPTNL) was recognized. This correlates with the lower antigenicity of the 7-mer and 5-mer synthetic peptides compared with that of the 9-mer. Thus, only IE1 antigenic sequences that as synthetic peptides were recognized at concentrations below 10⁻⁶ M were also detectable by CTL when presented from intracellularly synthesized proteins. Moreover, only such sequences could trigger a pp89-specific immune response *in vivo*. More generally, our results suggest the existence of an antigenicity threshold that must be reached for the presentation of antigenic peptides from intracellularly synthesized proteins. This may explain why some sequences are antigenic as synthetic peptides but are not recognized after intracellular antigen processing in presentation by major histocompatibility complex class I (3) or class II (4, 8) molecules.

The finding that a 9-mer sequence is sufficient for antigen presentation and induction of protective immunity agrees with recent data on naturally processed peptides isolated

from virus-infected cells, for which lengths of 8 and 9 aa have been found (21, 24). We suggest that the information obtained with synthetic peptides concerning the length and the antigenic potency of optimal antigenic sequences may have a predictive value also in other systems for the successful construction of recombinant vaccines containing short CTL epitope sequences.

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