

A NONSTRUCTURAL POLYPEPTIDE ENCODED BY  
IMMEDIATE-EARLY TRANSCRIPTION UNIT 1 OF MURINE  
CYTOMEGALOVIRUS IS RECOGNIZED BY CYTOLYTIC T  
LYMPHOCYTES

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Recovery of bone marrow transplant recipients from human cytomegalovirus (HCMV) infection is correlated with the presence of T lymphocytes (1). For a model system, we study the specific cellular immune response to murine CMV. Similar to acute HCMV disease in immunocompromised patients, immunodeficient mice succumb to MCMV infection (2). Because adoptively transferred MCMV-specific CTL have protective effects (2, 3), we set out to characterize the viral antigens recognized by CTL. MCMV has a large (235 kbp) nonsegmented DNA genome (4). Similar to herpes simplex virus (HSV) (5), gene expression in CMV is coordinated in a cascade fashion. The first viral transcripts are translated into immediate-early (IE,  $\alpha$ ) proteins that regulate the transcription of mRNA translated into early ( $\beta$ ) proteins, which in turn are required for the synthesis of late ( $\gamma$ ) proteins (6). Although MCMV IE proteins are nonstructural viral proteins (7), we observed that (a) CTL recognize antigen(s) expressed already during the IE phase (8), (b) IE-specific CTL make up the majority of MCMV-specific CTL (9), and (c) IE-specific CTL protect mice against lethal CMV disease.<sup>1</sup>

Genes coding for IE proteins are clustered in an 11-kb region (IE region) of the MCMV genome (6), comprising three transcription units (ie1, ie2, and ie3) (10). Transfection of the cloned IE region into L cells expressing the MHC L<sup>d</sup> gene led to antigen expression recognized by CTL (11). Here we report that L cell lines expressing the gene *ie1*, located in transcription unit ie1, are lysed by IE-specific CTL.

### Materials and Methods

*Plasmids and Selection of Stably Transfected Cell Lines.* The cloning of MCMV DNA fragments (4) and plasmids pAMB25, pMSVIE14, and pIE110 (6, 13) have been described before. Plasmid pIE100, which contains ie1, including the >700 bp MCMV enhancer

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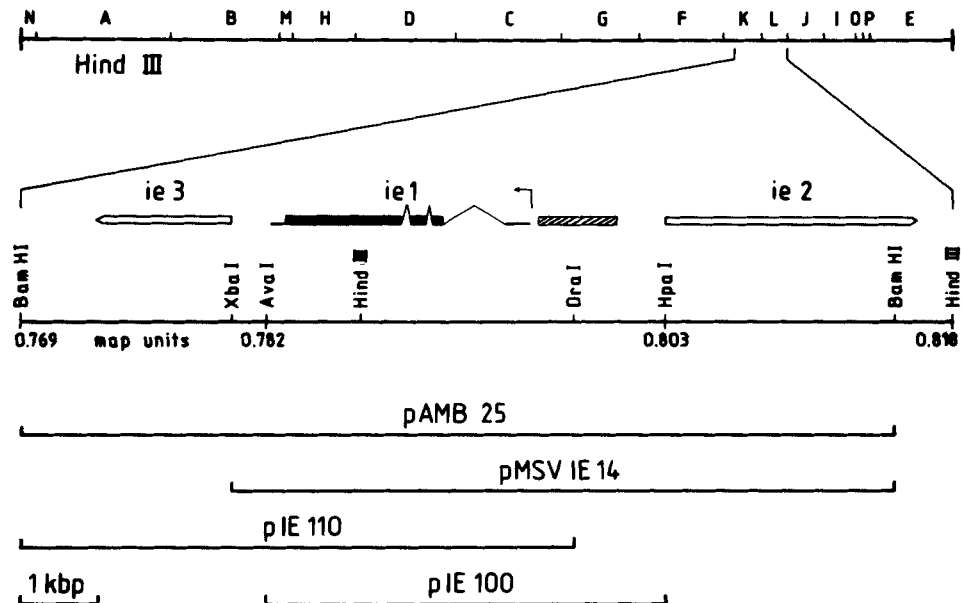


FIGURE 1. Physical map of the IE region of MCMV. *Top*: Hind III cleavage sites. *Center*: Location of transcription units, direction of transcription, and the structure of the major RNA transcribed from gene *ie1* in unit *ie1*. The segmented bar represents the open reading frame, and the hatched box indicates the promoter regulatory region. Units *ie2* and *ie3* are shown as open arrows to indicate the lack of precise knowledge of structural organization. *Bottom*: Plasmid-cloned MCMV DNA fragments used for transfection.

(14), was constructed as follows: The plasmid-cloned *ie1* was cleaved at the *Ava* I site located 29 bp downstream of the 3' end of the *ie1* gene in *ie1* (15), and *Hind* III linkers were added. The resulting 1215 bp *Hind* III fragment was reinserted into the unique *Hind* III site of *ie1*. The physical map of the IE region and plasmid-cloned DNA sequences are shown in Fig. 1. *Ltk*<sup>-</sup> cells were used for transfection. The neomycin resistance gene and the HSV *tk* gene served as selective markers. Colonies tested for IE antigen expression by indirect immunofluorescence (13) were cloned by limiting dilution. Expression of viral proteins was studied by radioimmunoprecipitation.

**Cytolytic Effector Cells.** CTL clones: Expression of MCMV IE antigen was tested with CTL clone IE1, line IE1.21-IL (12). MCMV IE antigen recognition by clone IE1 is *L*<sup>d</sup> restricted (11, 12). Expression of the *L*<sup>d</sup> gene was monitored with CTL clone B6 $\alpha$ *L*<sup>d</sup> (16). Polyclonal CTL: Specifically sensitized, IL-2-receptive CTL precursors (IL-CTLp) (9) were isolated from BALB/c mice 8 d after infection with 10<sup>5</sup> PFU of MCMV (strain Smith, VR-194; American Type Culture Collection, Rockville, MD). For the limiting-dilution assay, lymphocytes were propagated antigen-free for 8 d using recombinant human IL-2 (100 U/ml; Sandoz Forschungsinstitut, Vienna, Austria).

**Target Cells and Cytolytic Assay.** Mouse embryo fibroblasts (MEF) were infected with 20 PFU of MCMV per cell. Enhanced, selective expression of IE genes was achieved by infection in the presence of cycloheximide (50  $\mu$ g/ml) that was replaced after 3 h by actinomycin D (5  $\mu$ g/ml) to prevent the expression of  $\beta$  genes (6-9). Target cells (IE-MEF and transfected L cell lines) were tested in a standard 3-h <sup>51</sup>Cr-release assay. The estimation of precursor frequencies has been detailed before (9).

## Results

The T cell clone IE1 recognizes an epitope of an unknown IE gene product in association with *L*<sup>d</sup> (11, 12, 16). The epitope is detected after transfection of

TABLE I  
Recognition of an *ie1* Product by CTL Clone IE1

Construction of target cells			Percent specific lysis at clone IE1/target ratio of:				
IE transcription units	Plasmid	Line	10	5	2.5	1.25	0.6
ie3, ie1, ie2	pAMB25	L/IE.B25-L <sup>d</sup> /1	30.4	23.8	21.7	14.6	8.5
ie1, ie2	pMSV-IE14	L/IE14-L <sup>d</sup> /10	41.4	33.6	32.4	20.9	17.5
ie3, ie1	pIE110	L/IE110-L <sup>d</sup> /7	22.0	20.0	18.4	15.9	10.5
ie1	pIE100	L/ie1-L <sup>d</sup> /2	49.1	43.2	36.9	25.2	15.4

Data represent mean values of six replicate determinations. Clone IE1 did not lyse the transfectant line L/45/1, which expresses *ie1* but lacks L<sup>d</sup> (11, 13). Lysis by clone B6 $\alpha$ L<sup>d</sup> ranged between 24–69% at an effector/target ratio of 10:1.

the 10.8 kb Bam HI MCMV DNA fragment (plasmid pAMB25) into L cells that express L<sup>d</sup> (11) (Table I). This pAMB25 fragment contains more than one IE gene (10). The dominant IE mRNA, transcribed from gene *ie1* in unit *ie1*, is translated into the major IE protein pp89 (7). In addition, less abundant RNA species use the *ie1* start site, but are spliced differently. Some of these RNAs terminate in transcription unit *ie3* located downstream of *ie1* (10). A cell line transfected with plasmid pMSV-IE14 (*ie1*, *ie2*) DNA was recognized by the CTL clone, thus excluding a requirement of *ie3* sequences. A requirement of unit *ie2* was excluded by transfection of plasmid pIE110 (*ie3*, *ie1*). These data already strongly suggested that *ie1* encodes the antigen detected by clone IE1. This was proven by testing line L/ie1-L<sup>d</sup>/2. This line was transfected with plasmid pIE100 DNA, containing exclusively *ie1* DNA. Clone IE1 detected the antigen only when L<sup>d</sup> expression was sufficient for recognition by the L<sup>d</sup>-specific CTL clone B6 $\alpha$ L<sup>d</sup> (not shown).

Immunoprecipitation of viral proteins expressed in L/ie1-L<sup>d</sup>/2 revealed the presence of pp89 (Fig. 2a). The location of antigen was studied by immunofluorescence (Fig. 2b). The antigen was found to be diffusely distributed within the nucleus, sparing regions containing densely packed chromatin (Fig. 2c). There was no indication for membrane-associated or cytoplasmic antigen.

We further asked whether the epitope recognized by CTL clone IE1 in association with L<sup>d</sup> is the only IE epitope detected by the polyclonal CTL response of BALB/c mice to MCMV (Fig. 3). The cytolytic profile of CTL clone IE1 was identical on line L/ie1-L<sup>d</sup>/2 and on target IE-MEF that expressed all IE genes and all H-2<sup>d</sup> class I genes (Fig. 3a), demonstrating that the expression of the IE1 epitope was comparable in both targets. Nevertheless, the frequency of CTL specific for IE-MEF was found to be 1 in 6,500 lymphocytes, which is concordant with previous estimates (9), while the frequency of CTL that lysed L/ie1-L<sup>d</sup>/2 was 1 in 23,500 lymphocytes (Fig. 3b). Thus, CTL that detect the epitope defined by clone IE1 represent a substantial fraction (~25%) within polyclonal IE-specific CTL. In addition, CTL apparently exist that detect IE epitopes not presented in line L/ie1-L<sup>d</sup>/2.

## Discussion

We have identified the MCMV transcription unit that encodes the epitope recognized by an IE-specific CTL clone by using target cells that expressed the

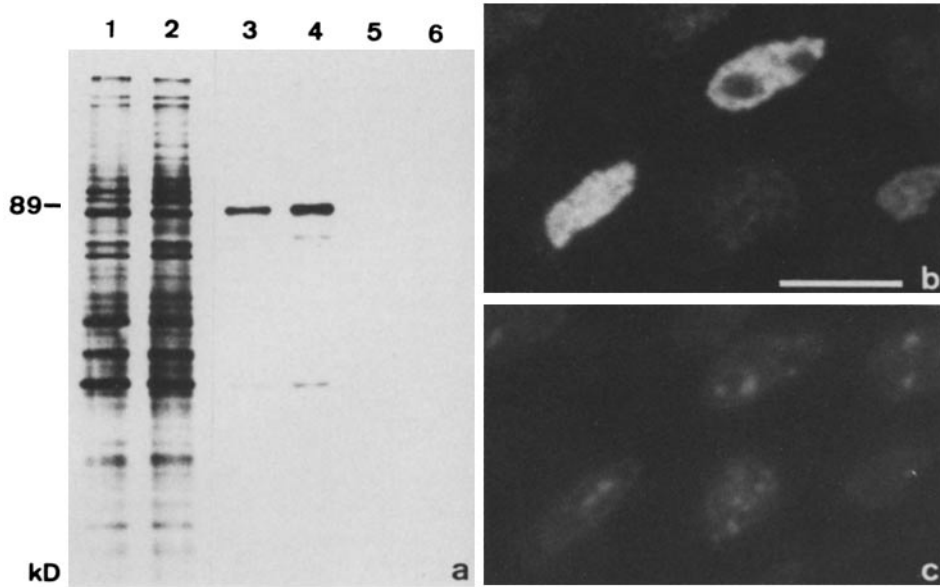


FIGURE 2. (a) Fluorogram of cellular polypeptides and immunoprecipitated proteins. Proteins labeled with [ $^{35}$ S]methionine were separated by 10% SDS-PAGE. Cellular polypeptides from L/ie1-L<sup>d</sup>/2 cells and L/L<sup>d</sup> cells are shown in lanes 1 and 2. Proteins from L/ie1-L<sup>d</sup>/2 cells precipitated by mAb 6/58/1 (lane 3) or by MCMV antiserum (lane 4), and from L/L<sup>d</sup> cells by mAb 6/58/1 (lane 5) or by MCMV antiserum (lane 6). (b) Indirect immunofluorescence on ultrathin section of L/ie1-L<sup>d</sup>/2 cells using MCMV antiserum and FITC-labeled goat anti-mouse IgG. The bar represents 10  $\mu$ m. (c) DNA staining of the identical section with DAPI (4',6-diamino-2-phenylindol dihydrochloride).

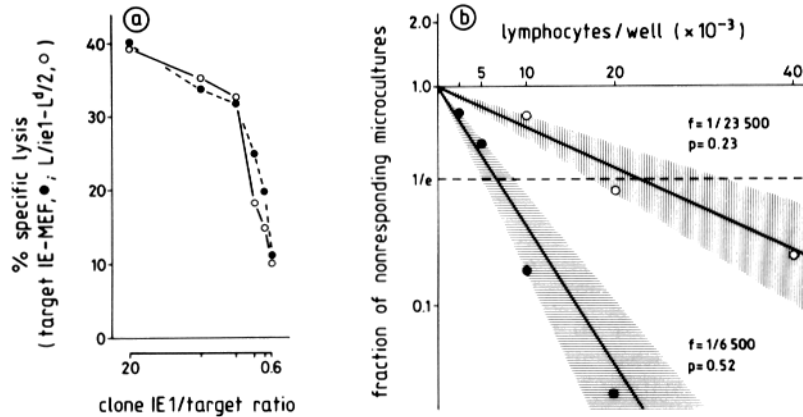


FIGURE 3. Proportion of polyclonal IE-specific CTL recognizing L/ie1-L<sup>d</sup>/2. (a) Lysis by CTL clone IE1 of IE-infected MEF targets and L/ie1-L<sup>d</sup>/2. (b) Limiting-dilution analysis of CTL recognizing IE-MEF (closed circles) and L/ie1-L<sup>d</sup>/2 (open circles). The hatched sectors represent the 95% confidence intervals of the frequency ( $f$ ) estimates (maximum likelihood method).

L<sup>d</sup> gene and MCMV IE proteins after gene transfection. Unit ie1 proved to be the only viral genomic region required.

The analysis of the polyclonal CTL response revealed that at least 25% of IE-

specific CTL recognize the L/ie1-L<sup>d</sup>/2 transfectant. Whether the other IE-specific CTL also recognize ie1 products presented in association with the K or D MHC class I genes or whether other IE genes encode additional epitopes remains open to question. In unit ie1 of MCMV, gene *ie1* codes for the major nonglycosylated phosphoprotein pp89 (7), a nonstructural protein active in transcriptional regulation (13). As expected for a regulatory protein, pp89 resides within the nucleus, and we could not detect this protein at the cell membrane. It is, however, premature to conclude that pp89 is processed to antigenic peptides, resulting in cell surface presentation, as has been postulated for the influenza virus nucleoprotein (17).

Recent detailed analysis of the IE transcription units has revealed that, in addition to the 2.75 kb mRNA translated into pp89, differently spliced smaller RNA species from ie1 are translated into proteins that are antigenically related to pp89 (10). Therefore, the question of whether pp89 by itself represents the antigen recognized by CTL is not yet answered. After having analyzed the structural organization of gene *ie1* (15), we plan to express the pp89 open reading frame selectively for final proof.

The major IE gene of HCMV is organized in a four-exon structure in the same manner as *ie1* of MCMV and codes for a protein similar in function (18). Our data propose the HCMV IE1 gene as the appropriate gene for testing the prediction of the murine model.

### Summary

We have constructed target cells by cotransfection of the MHC gene L<sup>d</sup> and fragments of murine cytomegalovirus (MCMV) DNA coding for nonstructural immediate-early (IE) proteins. Transfectants were tested by using CTL clone IE1 with specificity for an IE epitope presented in association with L<sup>d</sup>. Data show that clone IE1 recognizes a product of the ie1 transcription unit of MCMV, and that its specificity is shared by ~25% of polyclonal IE-specific CTL. The results provide the first definite evidence that expression of a herpes virus IE gene encoding a regulatory protein gives rise to antigen expression detectable by specific CTL.

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