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Host Immune Response to Cytomegalovirus: Products of Transfected Viral Immediate-Early Genes Are Recognized by Cloned Cytolytic T Lymphocytes

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To confirm that immediate-early (IE) genes of murine cytomegalovirus (MCMV) give rise to antigens recognized by specific cytolytic T lymphocytes (CTL), a 10.8-kilobase fragment of MCMV DNA which is abundantly transcribed at IE times was transfected into L cells expressing the L^d class I major histocompatibility glycoprotein. The viral genome fragment contains sequences of the three IE transcription units of MCMV: ie1, ie2, and ie3. In the transfected cell lines, only the predominant 2.75-kilobase transcript of ie1 and its translation product pp89 could be detected. The transfectants were analyzed for membrane expression of an IE antigen by employing clone IE1, an IE-specific CTL clone, as the probe. Only cells that expressed both the MCMV IE gene(s) and the L^d gene were recognized by the CTL clone.

Cytomegaloviruses (CMV), members of the herpesvirus family, cause severe disease only in immunodeficient hosts. The viral genome persists after infection, and reactivation can occur. The strict species specificity of CMV is a major constraint on the investigation of human CMV (HCMV) pathology. Murine CMV (MCMV) can be used as a model for studying the biology of CMV. In MCMV-infected immunodeficient mice, adoptive transfer of specific cytolytic T lymphocytes (CTL) protects against lethal disease (19). As with other herpesviruses, MCMV gene expression is sequentially controlled, giving rise to immediate-early (IE), early, and late proteins. Antigens detected by MCMV-specific CTL are already expressed during the IE phase (17), and frequency analysis revealed a high proportion of CTL specific for IE antigen(s) (18). The aim of this study was to identify the genomic region coding for an IE membrane antigen that can be recognized by CTL.

Transcriptional activity in the IE phase of infection maps to the *Hin*dIII fragments N, K, L, and E of the MCMV genome (7, 12). The region of abundant IE gene transcription is located between 0.769 and 0.817 map units (Fig. 1) (7). This region contains two major transcription units, ie1 and ie2. The predominant of 2.75-kilobase (kb) transcript of ie1 (transcribed from right to left according to the chosen presentation of the physical map) initiates at map position 0.796 and terminates about 700 base pairs downstream of the *Pst*I site (map position 0.785). This mRNA is translated into the major IE protein, pp89 (9), which activates promoters in *trans* (10). L/IE.45/1, a cell line selected after transfection of L cells with DNA sequences from map units 0.780 to 0.815, stably expressed pp89 (10). Analysis of the organization of the *ie*I gene revealed a four-exon structure. Numbers of exons and introns and the start of translation in exon 2 are analogous to the organization of the HCMV major IE gene (8a, 21). A further similarity between HCMV (22) and MCMV IE gene expression is the transcription of minor IE RNA species under control of the major IE gene promoter. The first exons of *ie*I are probably shared with the abundant RNA encoding pp89, but splicing occurs onto sequences downstream of ie1. Thus, a third coding region, ie3, can be defined. Region ie2 codes for a 1.75-kb mRNA translated in vitro into a 43,000-dalton protein. The transcription starts to the right of map unit 0.803 and terminates about 300 base pairs downstream of the *Bam*HI site at map unit 0.815 (8).

Availability of stably transfected cell lines expressing defined IE genes is one but not the only prerequisite for locating genes that specify IE antigens recognized by CTL. MCMV DNA fragments that contain IE coding regions could also code for not-yet-defined genes regularly expressed only during the early or late phases of viral replication. In transfected cells, in the absence of viral transcriptional control, however, such genes and IE genes could be expressed simultaneously. Further, it has been shown that CTL may detect a target antigen even when the protein concentration in transfected cells is too low to be detectable by immunoprecipitation (26). For these reasons, polyclonal CTL populations which contain not only CTL with specificity for IE antigens but also CTL with specificity for other virus-specified antigens could not be used as a specific probe.

For the identification of a particular IE membrane antigen we used line IE1.21-IL, an interleukin-2-dependent line derived from CTL clone IE1 (15). This clone defines an IE antigenic determinant recognized in association with class I glycoprotein encoded by the L^d gene of the murine major histocompatibility complex. This lytic activity of IE1.21-IL on IE-infected mouse embryo fibroblasts of the BALB/c strain ($H\text{-}2^d$) is shown in Fig. 2A (closed circles). As expected, this CTL line lysed neither the transfected L-cell line L/IE.45/1 ($H\text{-}2^k$) that synthesizes pp89 (10) but lacks the L^d glycoprotein (Fig. 2B) nor the L-cell line L/L^d that expresses the transfected L^d gene (14) but lacks viral genes (Fig. 2C). To provide both L^d and MCMV IE antigen, 10 μg of plasmid pAMB25 (Fig. 1) was cotransfected with 1 μg of the cloned *tk* gene as a selection marker into Ltk$^-$ cells transfected with the L^d gene (14). Colonies growing in selective medium were isolated and tested for IE antigen expression by indirect immunofluorescence with pp89-specific monoclonal antibody MCMV-6/58/1 (9) (Fig. 3a). Three pp89-positive colonies were grown to mass culture and tested for susceptibility

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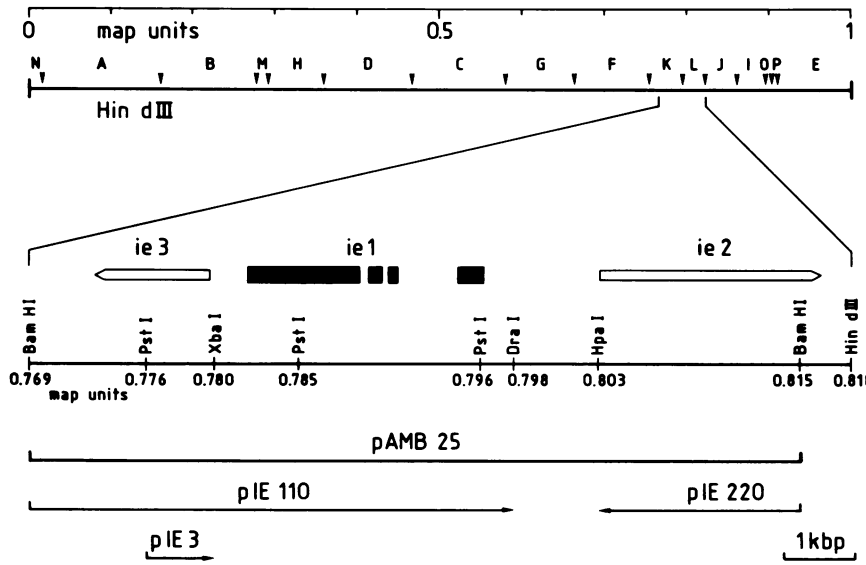

FIG. 1. Physical map of the genomic region of MCMV coding for abundant IE transcription. The genomic locations of *Hin*dIII cleavage sites (2) are shown (top). The region from 0.769 to 0.818 map units is expanded (below). The locations of the four exons of coding region ie1 encoding the 2.75-kb mRNA are shown as black boxes. The first small exon represents the 5′ end of the RNA. Coding regions ie2 and ie3 are shown as open boxes to indicate the lack of precise information on transcribed regions and gene structure. The 10.8-kb *Bam*HI fragment cloned in plasmid pAMB25 used for transfection is shown below. The three arrows (bottom) give the direction of transcription of complementary in vitro-synthesized RNA probes. The cloned genomic fragments (7, 8, 10) were transcribed after linearization of plasmids at the positions of the arrowheads.

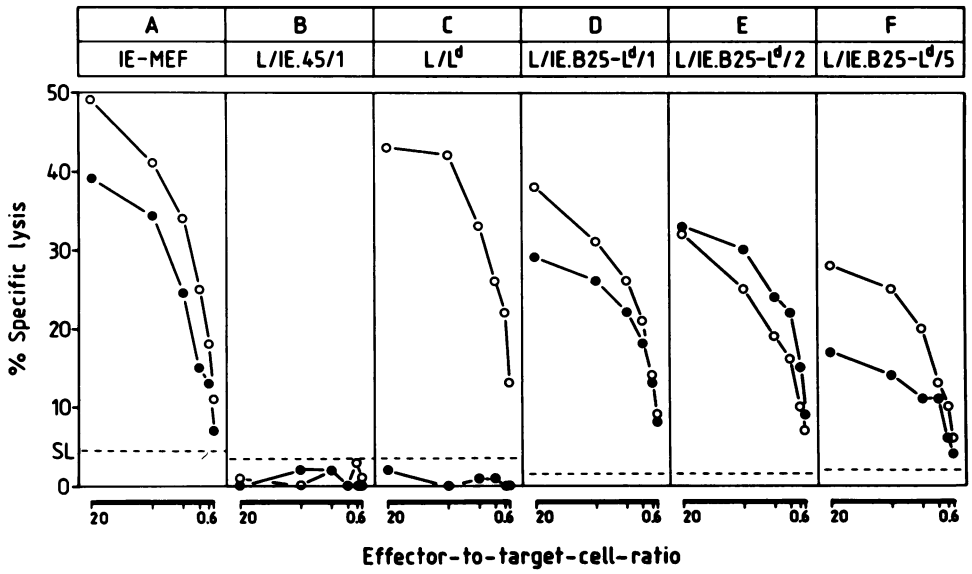

FIG. 2. Recognition of IE membrane antigen by CTL clone IE1 on L cells cotransfected with major histocompatibility complex class I L^d and MCMV IE genes. Subline IE1.21-IL of CTL clone IE1 was used to detect the IE1 epitope (15) (columns A to F, closed circles), and the cloned CTL line B6αL^d served as the control for the expression of the L^d glycoprotein (16) (columns A to F, open circles). For each of the indicated effector-to-target cell ratios (1,000 ^{51}Cr-labeled target cells), specific lysis was measured in a standard 3-h ^{51}Cr release assay. The mean values of six replicate determinations are given. The dashed lines represent the upper 95% confidence limit of spontaneous lysis taken as the significance limit. Columns: A, tertiary mouse embryo fibroblasts (BALB/c strain, L^d) were infected at a multiplicity of 20 PFU per cell with MCMV (ATCC VR-194, Smith strain) in the presence of cycloheximide (50 μg ml^{-1}) that was replaced after 3 h with actinomycin D (5 μg ml^{-1}) to achieve selective and enhanced synthesis of IE proteins (9); B, Ltk$^-$ cells (H-2^k) transfected with MCMV pMSV-IE14 (10); C, Ltk$^-$ cells transfected with the L^d gene (14) (kindly provided by H. Ponta, Kernforschungszentrum Karlsruhe, Federal Republic of Germany) D, E, and F, L/L^d cells transfected with MCMV pAMB25. The transfectant lines 1, 2, and 5 were tested in the cytolytic assay in the third in vitro passage 1 month after gene transfer.

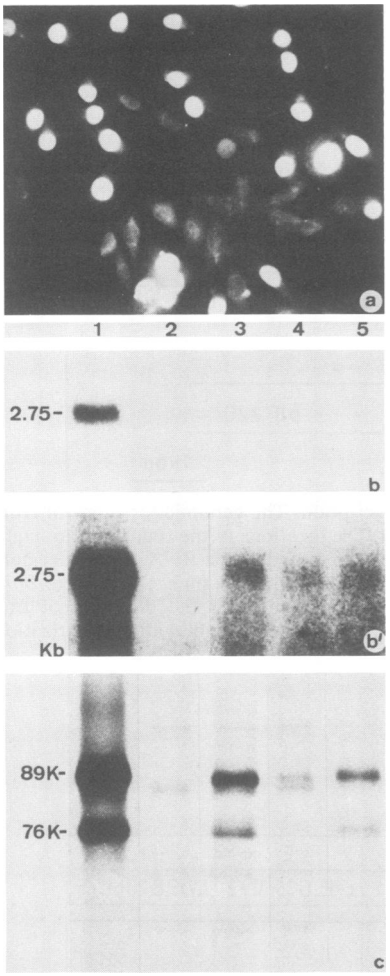

FIG. 3. Characterization of transfected L-cell lines. (a) MCMV IE antigen expression after transfection. Indirect immunofluorescence with monoclonal antibody MCMV-6/58/1 (9) shows nuclear expression of MCMV IE antigen in the transfected cell line L/IE.B25-L^d/1. (b) Northern blot analysis of RNA expressed in transfected cell lines. Total cellular RNA was extracted (7), and 0.5-μg portions (lane 1) or 5-μg portions (lanes 2 to 5) were fractionated on 1% agarose-formaldehyde gels and transferred to nitrocellulose paper. Hybridization was performed with in vitro-synthesized RNA transcribed from the plasmid pIE110 (Fig. 1). Hybridization conditions were as described before (7). Exposure to film was for 1 h (b) and 50 h (b'). The size of RNA is given in kilobases. Lanes: 1, (control) mouse embryo fibroblasts infected for 3 h with MCMV (multiplicity of infection, 50) in the presence of cycloheximide; 2, L cells transfected with L^d (L/L^d); 3 to 5, L/L^d sublines transfected with plasmid pAMB25: lane 3, line L/IE.B25-L^d/1; lane 4, line L/IE.B25-L^d/2; lane 5, line L/IE.B25-L^d/5. (c) Fluorogram of immunoprecipitated protein. Infected mouse embryo fibroblasts (multiplicity of infection, 50) and the transfected cell lines (panels b and b') were analyzed for expression of MCMV IE proteins. Fibroblasts were labeled with 1.5 mCi of [^{35}S]methionine ml^{-1} for 3 h after removal of cycloheximide and addition of actinomycin D. L-cell lines were labeled with [^{35}S]methionine for 8 h. Precipitated proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes are the same as in panel b.

to lysis by CTL and for expression of IE genes. Figure 2 shows the pattern of recognition by CTL line IE1.21-IL. All three transfected L-cell lines, L/IE.B25-L^d/1, -/2, and -/5 (Fig. 2D to F, closed circles), were recognized. The lysis was not significantly different from that found when infected fibroblasts which selectively expressed IE genes were used as target cells (Fig. 2A). Differences between the three cell lines with regard to the observed specific lysis were probably due to differences in the expression of the IE membrane antigen itself, since the lines were similarly susceptible to lysis by B6αL^d, a cloned L^d-specific CTL line (16) (Fig. 2D to F, open circles). Thus, a gene contained in the transfected MCMV sequences codes for the antigen defined by T-cell clone IE1.

Transcription of IE genes in the transfected cell lines was analyzed by Northern blot hybridization (Fig. 3b and b'). Hybridization of total cellular RNA with nick-translated pAMB25 indicated a transcript of about 2.75 kb only after very long exposure (data not shown). Hybridization with in vitro-synthesized RNA complementary to ie1 mRNA and ie3 mRNA transcribed by SP6 polymerase from pIE110 (Fig. 1) revealed a 2.75-kb RNA which proved to be the ie1 transcript (Fig. 3b and b'), since hybridization with RNA transcribed in vitro from pIE3 (Fig. 1) gave no detectable signal (data not shown). The level of IE gene transcription in transfectants was more than 2 orders of magnitude lower than in IE-infected cells (compare lane 1 and lanes 3 to 5 in Fig. 3b and b'). Plasmid pAMB25 lacks about 300 base pairs at the carboxy-terminal end of the ie2 coding region. This explains why transcription unit ie2, as also seen in previous transfections (10), did not give rise tp stable transcripts detectable by cRNA transcribed from pIE220 (Fig. 1) (data not shown). Immunoprecipitation with murine antiserum to MCMV corroborated these findings (Fig. 3c). Consistent with the expression of the 2.75-kb mRNA encoded by ie1, pp89 and its 76,000-dalton derivative (9) could be precipitated from the three transfectants. There seemed to be no correlation between the degree of specific lysis and the amount of mRNA or IE protein detected in the target cell lines. For instance, in that experiment, CTL recognition was best in line L/IE.B25-L^d/2 (Fig. 2E), although this line contained only low amounts of mRNA and pp89 (Fig. 3b' and c, lane 4). For technical reasons, cells used for the analysis of gene expression and cells that served as targets for the cytolytic assay were grown on separate dishes. Therefore, quantitative comparisons could not be made. When tested repeatedly, the transfectant L/IE.B25-L^d/1 turned out to be a target superior to L/IE.B25-L^d/2 (data not shown).

Altogether, the present study shows for the first time that a nonstructural herpesviral IE protein can be recognized by CTL after DNA transfection. It is most likely that pp89 or a derivative thereof is recognized by the CTL. However, considering the fact that T-cell recognition seems to be a more sensitive assay than immunoprecipitation, the possibility of recognition of an ie3 or a truncated ie2 product remains to be excluded.

The results contribute to recent reports that, contrary to earlier expectations, not only virion envelope glycoproteins, but also viral capsid proteins (3, 6), internal virion proteins (25–27), and nonstructural viral proteins (1, 4, 24) can serve as target antigens for CTL.

One question is how in the infected cell internal and nonstructural viral proteins are transported to the plasma membrane. MCMV pp89 is a nonstructural phosphoprotein located in the nucleus of the infected cell, and one of its

essential functions is the activation of transcription (9, 10). The deduced amino acid sequence of the protein (8a) does not predict membrane insertion domains, and hence the *ie*I gene product probably requires processing for cell membrane presentation. In addition, differently spliced minor mRNA species encoded by ie1 appear to be translated into protein (8). The question of whether a minor ie1 product is involved in target formation is under study.

Before we had mapped MCMV IE genes, we had used the term lymphocyte-detected IE antigen (17, 18) to indicate a potential similarity to another herpesvirus antigen recognized by CTL, the lymphocyte-detected membrane antigen of Epstein-Barr virus (13, 23). Recent work has indicated that the candidate for lymphocyte-detected membrane antigen, the latent infection membrane protein, contains transmembrane domains exposed on the outer cell surface (11). Our data provide evidence that the IE antigen(s) of MCMV and the Epstein-Barr virus antigen(s), with the tacit assumption that lymphocyte-detected membrane antigen is a part of the latent infection membrane antigen, are encoded by genes coding for proteins that have different functional properties and are expressed at the cell membrane by different mechanisms.

Another important question is why IE antigens dominate the CTL response to MCMV. Since during infection of permissive cells IE gene transcription is only transient, it is not surprising that herpesvirus IE gene products had previously not been taken into account as antigens eliciting cellular immunity. Two possibilities need to be considered. First, the expression of the IE membrane antigen is not necessarily synchronous with the regulatory function of pp89. Studies on the kinetics of IE antigen expression with CTL clone IE1 as a probe indeed revealed reexpression of the target antigen after viral DNA amplification during the late phase of the replicative cycle, concomitant with reinitiation of transcription from ie1 and new synthesis of pp89 (16). Second, and likely to be most important, the infection of permissive embryo fibroblasts in vitro does not necessarily reflect the duration of IE gene expression in other cell types infected in vivo. Some nonpermissive cell lines infected in vitro overproduce the major HCMV IE protein (5), and in vivo-infected cells from the blood of patients have been found to selectively express HCMV IE genes (20).

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