Cloned Long-Term Cytolytic T-Lymphocyte Line with Specificity for an Immediate-Early Membrane Antigen of Murine Cytomegalovirus

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Long-term cytolytic T-lymphocyte (CTL) lines that are specific for distinct antigens associated with different phases of the replicative cycle of the murine cytomegalovirus (MCMV) were established by cloning of CTL lines derived from lymph nodes of latently infected BALB/c mice. Two CTL clones were characterized in detail. Both displayed the Lyt-2^+ , L3T4^- surface phenotype, and the recognition of their respective target antigens was class I (DL^d) major histocompatibility complex antigen restricted. Clone S1 was specific for a structural antigen of MCMV, and clone IE1 detected an MCMV-specified immediate-early (IE) membrane antigen. Clone IE1 retained lytic activity, antigen specificity, and self-restriction after prolonged propagation in the presence of recombinant human interleukin-2 without restimulation by antigen. This interleukin-2-dependent line of the clone IE1, line IE1-IL, can serve as a reference line for the definition of the antigenic determinant IE1 of an IE membrane antigen.

The immediate-early (IE, α) genes of herpesviruses are the first viral genes transcribed by host cell RNA polymerase after infection of permissive cells (3, 8, 11, 25). The IE proteins perform a central regulatory role in the replication of herpesviruses. As exemplified for indicator genes, such as the E2 adenovirus early gene (9) and the human β -globin gene (6), herpesvirus IE proteins can activate other transcription units, and an IE protein (IE-Vmw175) of herpes simplex virus type 1 is required throughout the replicative cycle to promote the transition from IE to early (β) gene expression and the transcription of late (γ) genes (26).

Selective synthesis of murine cytomegalovirus (MCMV) IE proteins (12, 21) leads to the expression of at least one IE membrane antigen (21). A marked number of cytolytic T lymphocytes (CTL) activated during acute infection of mice (20) specifically lyse IE-infected indicator cells (22), and IE membrane antigen-specific memory CTL reside in lymphatic tissues during viral latency (21), suggesting that in the natural host the expression of IE genes is implicated in immune surveillance.

At present it has not been established whether only one or more IE membrane antigens exist, and molecules that carry lymphocyte-detected IE determinants have not yet been characterized biochemically. An 89-kilodalton phosphoprotein (pp89) located mainly in the nucleus and cytoplasm of the infected cell is the predominant MCMV IE protein (12). This protein in its authentic form or in a processed form and also additional IE polypeptides of low abundance (12) come into question as antigens. The identification of a particular IE membrane antigen requires a monospecific probe.

In this paper we describe the selection and characterization of the first cloned long-term CTL line specific for an IE membrane antigen of a herpesvirus. This line is compared with another cloned long-term CTL line specific for a structural (S) antigen of the same virus.

We have observed previously that BALB/c ($H-2^d$ haplotype) mice, infected intraperitoneally as newborns with

10^2 PFU of mouse embryo fibroblast (MEF)-adapted MCMV (ATCC VR-194, Smith strain), harbor memory CTL in lymphatic tissues when latency is established after a long period of persistent viral replication. Such memory CTL can be restimulated to effector CTL in vitro by adding infectious MCMV, and mesenteric lymph nodes turned out to be a superior source of memory CTL specific for IE membrane antigens (21).

Therefore, mesenteric lymph node lymphocytes derived from latently infected female BALB/c mice 10 months after primary infection were used for the cloning of CTL (started 10 July 1984). Lymphocytes (5×10^6) were restimulated in a 2-ml culture with 5×10^5 PFU of MCMV in the presence of 5×10^6 30-Gy γ -ray-irradiated splenocytes which were derived from latently infected, syngeneic donors to serve as antigen-presenting cells. The number of restimulations with antigen was recorded in the form of a code beginning with A1 to document the stage of the clones in later experiments. For cloning and long-term propagation of CTL, minimal essential medium alpha without nucleosides (catalog number 320-2561; GIBCO Laboratories) was used supplemented with 10% fetal calf serum, 4 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.2), 100 U of penicillin per ml, 0.1 mg of streptomycin sulfate per ml, 0.1 mg of kanamycin per ml, 2.5 μg of amphotericin B per ml, and occasionally, 1 μg of tylosin tartrate per ml. With the exception of the first restimulation, 50 U of interleukin-2 (IL-2; lymphokine supernatant of concanavalin A-activated rat splenocytes containing 500 U of IL-2 per ml) was added per ml. After the second restimulation, CTL were cloned by plating 0.5 lymphocytes per culture in round-bottomed 0.2-ml microtiter wells at a clone probability (17) of 77%, calculated under the premise that every cell is able to grow. The cloning microcultures had to be restimulated three times by refeeding with virus, antigen-presenting cells, and IL-2 until growing clones became visible (stage A5). The frequency of growing cells was then found to be 10^{-2} , and thus, the apparent clone probability was found to be $>99\%$.

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FIG. 1. Specific recognition of an IE membrane antigen and a virion S antigen by CTL clones. At stage A17, the specificity of cloned CTL lines was tested in a cytolytic assay. Selective synthesis of IE proteins in target IE MEF was achieved by infection of tertiary MEF (multiplicity of 2 PFU per cell) in the presence of cycloheximide (50 μ g/ml) that was replaced after 3 h by actinomycin D (5 μ g/ml). S MEF were prepared by mock infection with UV light (18,000 J/m² at 254 nm)-inactivated virus (S input with 2 and S high with 200 PFU equivalents per cell). For each of the indicated effector-to-target-cell ratios (1,000 ⁵¹Cr-labeled target cells), specific lysis was determined in six replicates in a standard 3-h ⁵¹Cr release assay. Mean values are normalized to the maximum specific lysis (33% for line IE1-A and 37% for line S1-A) observed in the presence of concanavalin A (ConA) (20 μ g/ml) at the highest effector-to-target-cell ratio tested.

Clones were transferred for expansion first to 48-well (1-ml) and finally to 24-well (2-ml) culture plates. Further propagation of the lines, designated A-lines (18) to indicate restimulation by antigen, was routinely done for >6 months by a twofold split of cultures every 6 days and restimulation of the subcultures.

The specificity of two cytolytic lines established that way, IE1-A and S1-A, was analyzed at stage A17 of in vitro propagation (Fig. 1). Tertiary MEF target cells exhibited either IE or S antigens. IE MEF expressed IE membrane antigens after enhanced, selective de novo synthesis of IE proteins, accomplished by infection in the consecutive presence of cycloheximide and actinomycin D (for the rationale of this procedure, see references 8, 21, and 22). The IE proteins expressed in these target cells were characterized by immunoprecipitation (12). S MEF carried only viral S antigens integrated into the target cell membrane from without by mock infection with UV light-inactivated, purified virions or by infection in the presence of actinomycin D (21, 22). Lectin (e.g., concanavalin A)-mediated lysis allows measuring the lytic potential of a test population regardless of the expression of relevant antigens on the target cells (22) and was used to define the maximum specific lysis (4).

With line IE1-A, the maximum cytolytic effect was ob-

served when IE MEF were used as indicator cells. Lysis was not detectable when MEF were mock infected with the same dose of inactivated virus (S input) and was still completely negative even when the dose was increased by 100-fold (S high). On the other hand, line S1-A lysed target S high MEF to the maximum possible degree. On target IE MEF and on the control target S input MEF, line S1-A detected the same low amount of a virion S antigen (now defined as the S1 antigen) that was integrated into the target cell membrane during penetration of the virions. The de novo synthesis of IE proteins in IE MEF did not contribute to cytolysis by line S1-A. With neither of the CTL lines could lysis be observed when inhibitor-treated, noninfected MEF were used as target cells (data not shown).

Thus, the cloned lines IE1-A and S1-A recognize their respective antigens in a mutually exclusive way. This result proves that the IE membrane antigen detected by clone IE1 is not among the structural antigens of the virion that become integrated into the target cell membrane during the process of penetration.

Lyt-2⁺ natural killer cells are also involved in immunity to MCMV infection and can be maintained as lines growing in IL-2 (1). It was therefore indispensable to verify that both clones belong to the lineage of self-restricted CTL to sustain

FIG. 2. Flow cytometric analysis of the surface phenotype of CTL clones. At stage A21, cells of the cloned lines were separated from dead feeder cells by Lymphoprep (Nyegaard, Oslo, Norway) density gradient centrifugation. The sandwich technique was applied for specific staining. For the first antibody, monoclonal antibody (mAb) anti-Lyt-2.2 (immunoglobulin G2a [IgG2a]) produced by hybridoma 19/178 (7), mAb anti-Lyt-1.2 (IgM) produced by hybridoma C3PO (16), or mAb GK1.5 (IgG2b) specific for the L3T4a determinant (4) was used. For the second antibody, fluorescein-conjugated affinity-purified F(ab')₂ fragments of goat anti-rat IgG (for mAb GK1.5) or goat anti-mouse IgG (Jackson Immuno Research, Avondale, Pa.) was used. Flow cytometric analysis was carried out on a fluorescence-activated cell sorter (FACS IV; Becton Dickinson FACS Systems, Sunnyvale, Calif.). The fluorescence signal was triggered on particles of size greater than platelets, and propidium iodide (2 µg/ml)-stained dead cells were excluded by electronic gating. Results are expressed on a log fluorescence scale with 10⁴ cells analyzed. The control profiles obtained by staining with second antibody alone are shown superimposed with finer contours only on the positive fluorescence histograms obtained with mAb anti-Lyt-2.2.

the conclusion that the CTL response discriminates between antigens associated with different phases of the viral replicative cycle.

Analysis of lymphocyte surface marker expression by flow microfluorometry, with syngeneic thymocytes as a positive reference to allow comparison of antigen densities (left vertical panel of fluorescence histograms in Fig. 2), revealed that both clones, S1-A as well as IE1-A at stage A21 (about

4 months) of in vitro propagation, expressed the Lyt-2.2 antigen (upper horizontal panel of fluorescence histograms in Fig. 2), whereas the Lyt-1.2 antigen and the L3T4 (MT4) antigen (4) were not detectable. In conclusion, both cytolytic clones belong to the Lyt-2⁺, Lyt-1⁻, L3T4⁻ subset of T lymphocytes.

To determine whether the CTL lines recognize their respective MCMV-specified antigens in association with

TABLE 1. Class I MHC antigen restriction of cloned MCMV-specific CTL lines^a

Effector cells	Protocol code	Target cells (% specific lysis at E/T ratios of 5:1 and 1.25:1) from line:			
		KD2SV (K ^d DL ^d) IE/S/ni	B6/WT-3 (K ^b DL ^b) IE/S/ni	KHTGSV (K ^d DL ^b) IE/S/ni	K5RSV (K ^b DL ^d) IE/S/ni
Clone S1-A	A31	—/67-35/2-1	—/2-1/0-2	—/0-0/1-0	—/27-12/0-1
Clone S2-A	A27	—/33-22/12-0	—/2-2/2-0	—/4-1/1-0	—/38-25/12-3
Clone S3-A	A31	—/36-25/0-0	—/1-1/0-0	—/0-0/0-0	—/0-1/0-0
Clone IE1-A	A31	58-30/4-1/0-0	2-0/0-0/0-0	0-0/0-2/0-0	31-15/2-0/0-0
Clone IE1-IL	A22IL9	65-29/0-0/0-0	0-0/1-0/1-0	4-0/0-0/0-0	18-4/0-0/0-0
Polyclonal H-2 ^b anti-H-2 ^d /A2		—/—/57-41	—/—/6-4	—/—/49-31	—/—/31-19
Polyclonal H-2 ^d anti-H-2 ^b /A2		—/—/7-2	—/—/49-24	—/—/29-17	—/—/33-12

^a Simian virus 40-transformed cell lines were derived from C57BL/6 MEF (19) (line B6/WT-3) or from kidney tissue of congenic B10 strains (14) B10.D2 (line KD2SV), B10.HTG (line KHTGSV), and B10.A(5R) (line K5RSV). The lines were used as target cells after either MCMV infection (multiplicity of 20 PFU per cell) under conditions of selective IE gene expression (IE) or integration of high amounts (200 PFU equivalents per cell) of MCMV S proteins (S), or noninfected (ni) cells were used. Note that S antigens also became integrated into IE target cells and could be detected by S-specific CTL (see Fig. 1). For the sake of clarity, these data are not included. As in Fig. 1, lytic activity was determined in six replicates for various effector-to-target-cell (E/T) ratios. Representatively, the mean value of specific lysis is given without normalization only for two E/T ratios.

class I glycoproteins encoded by genes of the *K* or *DL* loci of the murine major histocompatibility complex (MHC) (13, 27), simian virus 40-transformed cell lines (14) derived from the congenic recombinant strains B10.HTG (line KHTGSV, allele combination *K^dDL^b*) and B10.A(5R) (line K5RSV, allele combination *K^bDL^d*) served as target cells after integration of MCMV S proteins or expression of MCMV IE membrane antigens. The expression of class I antigens by the indicator cell lines was controlled with alloantigen-specific CTL populations (Table 1).

Antigen recognition by the CTL lines S1-A and IE1-A proved to be *DL^d* restricted, and noninfected indicator cells were not lysed. This observation implied that these two MCMV-antigen-specific CTL lines did not recognize minor histocompatibility antigens of the B10 strains or any of the antigenic sites (2) of the simian virus 40-specified tumor-specific transplantation antigen that is expressed in the transformed cell lines used and can be detected by CTL (2, 5, 14).

Another *DL^d*-restricted CTL line with specificity for MCMV S antigen, line S2-A (Lyt-2⁺, Lyt-1⁻, L3T4⁻), showed at least some *DL^d*-associated activity against noninfected target cells, and for line S3-A (Lyt-2⁺, Lyt-1⁻, L3T4⁻), restriction could not be mapped to an MHC class I locus.

At restimulation stage A18, lymphokine supernatant as a source of IL-2 was replaced by recombinant human IL-2 (lot 89050/84802; >99% pure IL-2; specific activity, 7.3×10^6 U per mg of protein; Sandoz Research Institute, Vienna, Austria). After stage A22, line IE1-A was split, and a subline was further propagated as line IE1-IL by passaging in 6-day intervals in 50 U of recombinant human IL-2 per ml without antigen or feeder cells. At stage A22IL9, line IE1-IL was found to be strictly IL-2 dependent (data not shown) and to have retained lytic activity, antigen specificity, and MHC restriction (Table 1). Meanwhile (20 July 1985), several stable subclones of line IE1-IL have been established by repeated recloning and could be maintained for >7 months by continuously growing in recombinant human IL-2.

In conclusion, we report the selection of a monoclonal IL-2-dependent CTL line specific for an IE membrane antigen expressed by cells as a result of selective synthesis of MCMV IE proteins. Since T lymphocytes possess a receptor with idiotype specificity (10), clone IE1 can be used to define the antigenic determinant IE1 of an IE membrane antigen. The predominant IE protein is a nonglycosylated phosphoprotein (pp89) and is located in the nucleus and cytoplasm of the infected cell (12). The finding that lysis by clone IE1 could not be inhibited with either polyspecific murine antiserum or IE pp89-specific monoclonal antibody MCMV-6/20/1 (M. J. Reddehase and M. R. Fibi, unpublished observations) is in line with the apparent intracellular localization of pp89 but cannot be considered a formal proof against surface localization of a minor fraction of native or modified pp89. Recognition of fibroblasts by CTL was also observed after gene transfer of an influenza A virus gene coding for the nucleoprotein which is a nonglycoprotein component of the virion (24). Although MCMV IE proteins are not components of the virion, the analogy between influenza A virus infection and MCMV infection, with respect to the involvement of intracellular nonglycoproteins in recognition of infected cells by CTL, points to a more general phenomenon concerning antigen presentation by virus-infected cells. Since fibroblasts are capable of antigen processing and can present peptide fragments of proteins to T cells, provided that the relevant MHC gene products are

expressed (15), processing products of IE proteins also could serve as antigens presented in association with the MHC-encoded class I glycoproteins. The line IE1-IL provides the basis for studying the expression of the epitope IE1 after transfection of cells with authentic or mutated IE genes and will aid molecular genetic and biochemical analysis in defining the nature of the lymphocyte-detected IE antigen.

A therapeutic effect of CTL on established MCMV pneumonia has already been demonstrated with a polyclonal population (23). A possible implication of IE membrane antigens in the immune control of viral replication in tissues of an infected host can now be evaluated with monoclonal probes.

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