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Infection of APC by Human Cytomegalovirus Controlled Through Recognition of Endogenous Nuclear Immediate Early Protein 1 by Specific CD4+ T Lymphocytes

Emmanuelle Le Roy,* Michel Baron,* Wolfgang Faigle,† Danièle Clément,* David M. Lewinsohn,§ Daniel N. Streblow,§ Jay A. Nelson,§ Sebastian Amigorena,† and Jean-Luc Davignon2*

Infections by human CMV are controlled by cellular immune responses. Professional APC such as monocytes and macrophages can be infected in vivo and are considered as a reservoir of virus. However, CMV-specific CD4+ responses against infected APC have not been reported. To develop a model of CD4-infected APC interaction, we have transfected the U373MG astrocytoma cell line with the class II transactivator (CIITA). Confocal microscopy experiments showed that U373MG-CIITA cells expressed markers characteristic of APC. Functional assays demonstrated that infected U373MG-CIITA APC processed and presented both exogenous and endogenously neosynthesized nuclear immediate early (IE) protein 1 through the MHC class II pathway. More importantly, endogenous presentation of IE1 by infected APC lead to efficient control of CMV infection as revealed by decreased viral titer. Thus, these results describe the endogenous presentation of a nuclear viral protein by the MHC class II pathway and suggest that IE1-specific CD4+ T cells may play an important role in CMV infection by directly acting against infected APC. The Journal of Immunology, 2002, 169: 1293–1301.

Cellular immune responses appear to control infection by β herpesvirus CMV. Primary infections are usually mild and asymptomatic and during latency, subsequent to primary infections, no CMV Ag expression or clinical manifestation can be observed. Severe CMV disease is the consequence of immune deficiency (1). Thus, the study of recognition of CMV Ags and control of CMV infection has been of special interest (for review, see Ref. 2). Despite numerous viral escape mechanisms (reviewed in Refs. 3 and 4), CD8+ T cells specific for CMV expand in CMV seropositive individuals (5–8) and are able to kill infected targets in vitro (9). Cell therapy based on the injection of matrix Ag-specific CD8+ T cell clones prevents CMV disease in bone marrow-transplanted patients (10). An increase of cytotoxic cells in acute CMV infections coincides with recovery (11). Thus, strong arguments are in favor of a critical role of CD8+ T cells in the control of acute and latent CMV infections.

Additionally, a high frequency of CD4+ T cells specific for CMV Ags has been widely reported (12–15). Ag specificity has been shown against several proteins such as immediate early (IE) protein 1, IE2, glycoprotein B (gB), and phosphoprotein (pp) 65, either at the population (16–18) or the clonal (19, 20) level. Anti-IE1 specificity of CD4+ T cells has been of particular interest (12, 19, 21) because it is highly expressed a few hours after the start of infection and throughout the replicative cycle (22). Compared with CD8+ T cells, the functionality of anti-CMV CD4+ T cells has been less explored. Riddell and Greenberg (23) have shown a role in vivo for recovery of CD4+ T cell function in protection from CMV disease after bone marrow transplantation. Data from bone marrow-transplanted patients injected with anti-CMV CD8+ T cells clones suggest that maintenance of CD8+ T cells depends on recovery of endogenous CD4+ T cell function (10). There is an association between anti-pp65 CD8+ T cell response and anti-CMV CD4+ T cell response in HIV infection (24). End organ CMV disease in AIDS is correlated with poor CD4+ T cell response to CMV (25). Direct anti-CMV functionality of CD4+ T cells has been suggested by experiments which showed that supernatants from IE1-specific cloned CD4+ T cells activated with soluble Ag inhibit CMV replication due at least in part to secreted IFN-γ and TNF-α (19). But the responses of CMV-specific CD4+ to, and functional consequences on, CMV-infected APC have not yet been documented.

The possible recognition of infected APC by CD4+ T cells is of importance because monocytes and macrophages can be infected and are considered as cell reservoirs of virus that reactivates CMV upon cell differentiation (26, 27). Dendritic cells have also been recently reported to be permissive (28–30). Because analysis of presentation and consequences of recognition of infected APC by CMV-specific CD4+ T cells have not yet been investigated, we have stably transfected permissive U373MG astrocytoma cells with the cDNA encoding for the MHC class II (MHC-II) transactivator (CIITA) to construct a cell model of Ag presentation (31). U373MG cells were chosen because they have mulosis; MOL, multiplicity of infection; GFP, green fluorescent protein; Li, invariant chain; Lamp, lysosome-associated membrane protein.
been shown to be productively infected by CMV (19, 32) and astrocytes have been shown to be able to present Ag (reviewed in Ref. 33).

In this study, we used the model of U373MG-CIITA cells to investigate the recognition of infected APCs by IE1-specific CD4+ T cell clones and the effect on CMV infection. We found that IE1-specific CD4+ T cells were efficiently activated by infected U373MG-CIITA cells through endogenous presentation. This activation resulted in IFN-γ production by and proliferation of IE1-specific CD4+ T cell clones. Interaction of IE1-specific IE1 T cells led to efficient control of infection. We conclude that recognition of infected APCs can be performed by IE1-specific CD4+ T cells and that this interaction may be of importance in the control of CMV infections in vivo.

Materials and Methods

Cell lines, CD4+ T cell clones

Culture media were RPMI (for tumor cells and CD4+ T cells) and DMEM (for MRCS cells) supplemented with sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and glucose (2 mM; Life Technologies, Cergy Pontoise, France).

Human astrocytoma cells U373MG were a kind gift from S. Michelson (Institut Pasteur, Paris, France). EBV-transformed B lymphoblastoid cell line (Steinlin) was from the 10th Histocompatibility Workshop (New York, NY; B. Dupont). Both were cultured in culture medium supplemented with 10% FCS. U373MG-CIITA cells were obtained as described (31) by transfection of U373MG with the pSRANeo/CIITA plasmid and were cloned by limiting dilution.

IE1-specific CD4+ T cell clones, FzD11, FzD3, HLA-DR3-restricted, and HeA10, HLA-DR7-restricted, which have been reported (19), were generated using an ECL kit (Amersham Biosciences). Supernatants in an ELISA using a pair of IFN-α2b mAbs from Medogenix (Les Ulis, France). After collecting supernatants, wells were reseeded in 96-well plates at the same concentration as U373MG-CIITA cells. In both cases, supernatant was collected after 24 h of culture.

Kinetics of IE1 protein expression postinfection were measured using an IE1-specific rabbit polyclonal Ab (12), followed by a rhodamine-coupled goat anti-rabbit antisemur (Beckman Coulter). Slides were examined under a Leitz Axiosphot fluorescence microscope (Leica).

Detection of IE1 by immunoprecipitation and Western blotting

One milliliter of CMV Towne strain corresponding to 108 PFU was ultracentrifuged at 100,000 × g for 30 min. The viral pellet was resuspended in 1 ml of PBS and ultracentrifuged in the same conditions. The step was repeated three times. Supernatants were kept for further analysis. The final pellet was resuspended in 1 ml of lysis buffer (50 mM Tris, pH 7.5, 10 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 10% glycerol, 1% Triton X-100). All fractions (virus lysate and supernatants) were precleared with zyozorine (Zymed Laboratories, Montegue, France) for 30 min at 4°C. E13 mAb was added for 1.5 h at 4°C, followed by protein G-Sepharose beads (Sigma-Aldrich, Saint-Quentin Fallavier, France). Incubation was performed overnight at 4°C. The beads were washed four times with PBS and resuspended in SDS sample buffer. The presence of IE1 protein was tested by Western analysis on a 10% acrylamide/SDS gel using mAb E13 as the primary Ab and peroxidase-coupled rabbit anti-mouse Ig as the secondary Ab (Amersham Biosciences, Orsay, France). Detection was performed using an ECL kit (Amersham Biosciences).

Ag presentation by U373MG-CIITA cells

U373MG-CIITA and control U373MG cells were seeded (4 × 105 cells/well) in flat-bottom 96-well plates, then pulsed overnight with Ag (peptide or IE1-p66 protein; Ref. 14). Cells were then washed twice, fixed with 0.5% glutaraldehyde (Sigma-Aldrich) for 1 min, washed three times and incubated with the IE1-specific FzD11 CD4+ T cell clone (2 × 105 cells/well). The same procedure was used with EBV-B cells except that they were pulsed (5 × 105 cells/ml) with Ag in 24-well plates and subsequently seeded in 96-well plates at the same concentration as U373MG-CIITA cells. In both cases, supernatant was collected after 24 h of culture.

Viruses

CMV (Towne) stocks were obtained by infection of MRCS cells (bioMérieux, Charbonnier les Baines, France) at a multiplicity of infection (MOI) of 0.1 in 10% FCS culture medium. CMV HV5-111 Toledo strain that expresses green fluorescent protein (GFP) under the control of the cellular elongation factor 1α (35) was propagated in human dermal fibroblast cells (NHDF).

Towne strain virus titration was performed, as described, on MRCS cell line (Aventis Pasteur, Lyon, France). Titration of CMV HV5-111 was also performed on MRCS by counting fluorescent cells under an inverted fluorescence microscope.

Antibodies

Anti-HLA-DR Abs were I-B5 (36) and L243, (from American Type Culture Collection, Manassas, VA). Anti-invariant chain (li) was Bu45 (37). Anti-liposome-associated membrane protein (Lamp)-1 mAb was from BD PharMingen (San Diego, CA). Rabbit anti HLA-DM antiserum was made against a cytosolic peptide of HLA-DM β-chain.

Anti-CMV protein mAbs were: E13, an IgG1 specific for UL122 and 123 (a kind gift from M.-C. Mazeron, Hôpital Lariboisière, Paris, France), 1201, an IgG1 specific for gB, (from the Goodwin Institute for Cancer Research, Plantation, FL), CCH2, an IgG1 specific for pp52 (DAKO, Trappes, France).

Secondary Abs TR-conjugated donkey anti-mouse F(ab')2, and TR-conjugated donkey anti-rabbit F(ab')2, were purchased from The Jackson Laboratory (Bar Harbor, ME). PE-conjugated goat anti-mouse F(ab')2, were purchased from Beckman Coulter (Fullerton, CA).

Fluorescence microscopy

U373MG-CIITA cells were grown on sterile glass coverslips (5 × 104 cells/coverslip) and put in six-well plates (Falcon; BD Biosciences, Le Pont de Claix, France) in 10% FCS culture medium. When they reached 50% confluence, cells were fixed in methanol for 1 h at ~20°C, then incubated with PBS containing BSA (0.2%) and saponin (0.05%) for 20 min (washing buffer).

Staining procedure. Cells were incubated with the first-step Ab (anti-li, anti-DM, anti-Lamp-1, anti-IE1 + IE2 or anti-IE1) for 30 min, then washed twice with staining/washing buffer. Cells were then incubated with the Texas Red-coupled secondary Ab for 30 min, then washed twice. HLA-DR staining was then performed using FITC-coupled 1B5 mAb. Coverslips were mounted on slides with mowiol and slides were analyzed by confocal microscopy (TCS microscope; Leica, Heerberg, Switzerland).

Cytotoxicity of CD4+ T cells

CD4+ T cells were efficiently activated by infected U373MG-CIITA cells at a MOI of 5. Virus was incubated with cells for 1 h, cells were washed, and CD4+ T cell clones (300,000 cells/well) were added. Experiments were done in duplicate. Cells were collected on
day 6 postinfection and an aliquot was used to count cells using a Neubauer cell chamber. Samples were lysed by sonication, and serial dilutions of cell lysates were used for virus titration.

To test for cell-cell interaction, U373MG-CIITA and control U373MG cells were seeded in duplicate in 24-well plates (50,000/well) and, separately, in 3-μM pore polycarbonate membrane 10-mm diameter inserts (Nunc, Naperville, IL). The following day, CMV inoculum was added to cultures, incubated for 1 h with cells, washed away, FzD11 clone (200,000 cells/well) was added, and inserts were immediately placed above the cell layer of culture well.

Alternatively, U373MG and U373MG-CIITA cells were seeded (100,000 cells/well) in duplicate in 24-well plates. The following day, cells were incubated for 1.5 h with soluble IE1-free CMV, washed twice and FzD11 CD4+/H11001 clone (300,000 cells/well) was added to the culture. Cells were recovered after 6 days of culture, lysed by sonication, and virus titer was measured.

Flow cytometry

Intracellular CMV Ag detection. Intracellular expression of pp52 was analyzed as described (8) by flow cytometry. U373MG-CIITA cells were fixed/permeabilized in methanol at −20°C for at least 1 h, washed in staining buffer, and incubated with pp52-specific CCH2 mAb or IE1 + 2-specific mAb E13 for 1 h at 37°C, washed twice, and further incubated with PE-conjugated goat anti-mouse F(ab')2. After two washes, cells were analyzed using an EPICS Elite cell sorter (Beckman Coulter).

Cell counting by flow cytometry. Cells were harvested and counted using calibrated fluorescent beads (TruCount tubes; BD Biosciences) by flow cytometry (Epics Coulter Elite; Beckman Coulter). Beads and cells could be easily distinguished in separate windows according to size and fluorescence. Electronic events were counted in each separate window. The number of live U373MG and U373MG-CIITA cells harvested was calculated according to the following formula: number of target cells (U373MG or U373MG-CIITA)/tube (i.e., harvested) = (number of events/cell window) × (total number of beads/tube)/number of beads in fluorescence window).

Results

Immunofluorescence analysis of the expression of proteins involved in Ag presentation in U373MG-CIITA cells

Confocal microscopy analysis of proteins involved in Ag presentation was performed in a typical clone of U373MG-CIITA cells (Fig. 1).

Transfection of the cDNA encoding for CIITA into U373MG cells induced the constitutive expression of HLA-DR and li in U373MG-CIITA cells. Colocalization of these molecules was only partial as evidenced from the yellow color obtained in the presence of both markers. Likewise, HLA-DM expression was also induced and colocalization with HLA-DR was observed (Fig. 1B), although it was partial, as in the case of li and HLA-DR. The expression of Lamp-1, a marker of late endosomes and lysosomes, was also found to partially colocalize with HLA-DR (Fig. 1C). HLA-DR expression was observed both in the cytoplasm and cell surface (Fig. 1, A–C). As expected, stainings of untransfected U373MG cells consistently showed no expression of HLA-DR, DM, and li (data not shown). To ascertain surface expression of HLA-DR, a flow cytometry experiment confirmed that HLA-DR was strongly expressed at the cell surface of U373MG-CIITA cells (data not shown).

Presentation of exogenously added IE1 by U373MG-CIITA cells

To evaluate the ability of U373MG-CIITA cells to present Ag, we compared activation of the HLA-DR3-restricted IE1-specific FzD11 CD4+ T cell clone in the presence of Ag-pulsed U373MG-CIITA and HLA-DR3-expressing EBV-transformed B cells. Fig. 2 shows that IFN-γ production by FzD11 in response to presentation

FIGURE 1. Immunofluorescence analysis of U373MG-CIITA cells. U373MG-CIITA cells were double stained for HLA-DR and li (A), HLA-DR and DM (B), and HLA-DR and Lamp-1 (C) expression and analyzed by confocal microscopy. U373MG-CIITA cells were infected for 6 h and double-stained for HLA-DR and IE1 + 2 proteins (D).
of IE1 (91–110) peptide by U373MG-CIITA and EBV-B cells was equivalent. However, when IE1 protein was used as Ag, U373MG-CIITA cells where more efficient APC than EBV-B cells: concentrations of Ag required for similar IFN-γ production by FzD11 were 10- to 100-fold higher using EBV B cells than using U373MG-CIITA cells. Therefore, U373MG-CIITA behaved as potent APC. This reflected stronger Ag processing capacities of U373MG-CIITA cells compared with EBV-B cells. As expected, no activation of CD4+ FzD11 cells was observed using uninfected U373MG cells (not shown).

**Presentation of IE1 by infected U373MG-CIITA cells**

U373MG-CIITA cells were infected with CMV to test whether endogenously synthesized IE1 could be presented by HLA-DR. U373MG-CIITA were pulsed with CMV for 1 h, then washed, and infection and Ag processing were allowed to proceed. Double staining for HLA-DR and IE1/IE2 Ags showed separate localization of these markers (Fig. 1). As reported (38, 39), IE1 and IE2 staining for HLA-DR and IE1/IE2 Ags showed separate localization. Supernatant from cultures with infectious virus was used as a control for the presence of IE1 released in the culture medium. Untreated U373MG-CIITA cells were used as negative control. Activation of CD4+ T cell clone FzD11 in the presence of infected U373MG-CIITA was analyzed by proliferation (A) and IFN-γ production (B) after various times of infection. Numbers 1, 2, and 3 refer to the different phases of presentation during infection of U373MG-CIITA cells.

**Control of infection by IE1-specific CD4+ T cells**

We had previously shown that supernatant from activated IE1-specific CD4+ T cell clones could control infection of U373MG cells (19). In the present series of experiments, we tested whether CD4+ T cells specific for IE1 were capable of controlling CMV infection upon recognition of APC. We first used crude preparations of CMV to infect APCs.

To quantitatively assess the inhibition of CMV protein expression in infected U373MG-CIITA cells, we measured the expression of pp52 by flow cytometry. This protein is the product of UL44, the viral DNA polymerase accessory protein. It has been shown to correlate well with the intensity of cell infection in vitro (19). The influence of the CD4+ T cell/infected APC ratio was investigated. As shown in Fig. 4, the addition of increasing...
amounts of FzD11 clone strongly inhibited the expression of pp52 in infected U373MG-CIITA cells, whereas the expression of pp52 remained stable in control U373MG cells. Although control of pp52 expression could occur in the absence of cytotoxicity (10,000–100,000 cells of CD4+ clone), we observed that, at the highest concentration of FzD11 cells (300,000 cells), cytotoxicity was observed in addition to decreased CMV protein expression. This was evidenced by a decreased number of cells in the gate used for flow cytometry analysis of experiments such as that described in Fig. 4 (data not shown). Immunohistochemistry experiments showed that gB expression was diminished in the presence of IE1-specific CD4+ T cell clone, suggesting that the control of infection was still effective at the late phase of infection (data not shown).

We thus sought to evaluate the consequences on CMV production and the specificity of control of infection. U373MG-CIITA cells were incubated with CMV, then CD4+ T cell clones of different specificities were added to the cultures. CMV Toledo-GFP strain was used in this experiment to investigate both the ability of CD4+ T cells to control infection by a CMV clinical isolate and to visualize infected cells by fluorescence. Fig. 5 shows the results expressed both in cell numbers (Fig. 5A) and PFU/ml (Fig. 5B) in different conditions of culture. In accordance with Refs. 19 and 31, U373MG cells were capable of productive infection by CMV. The number of U373MG-CIITA cells was reduced 10-fold in the presence of high concentrations of FzD11 T cell clone compared with control cultures in the absence of CD4+ T cell clone. This logically resulted in lower CMV titers obtained from cell lysates of U373MG-CIITA of the same cultures. An IE1-specific CD4+ T cell clone HeA10, restricted by a different HLA-DR (HLA-DR7; Ref. 19) gave similar data as those observed in control cultures in the absence of CD4+ T cell clone. Similarly, the control in the presence of H9, a Tb Ag-specific HLA-DR3-restricted CD4+ T cell clone (34), in the absence of Ag showed data similar to the control in the absence of the CD4+ T cell clone. When Tb Ag was added to the culture of the H9 CD4+ T cell clone, however, a slight decrease both in the cell number and the CMV titer was observed. This may be due to the response and activation of H9 to presentation of Tb Ag by infected cells. Control experiments using U373MG cells showed, as expected, no decrease in numbers of cells and PFU. Therefore, recognition of infected APC by IE1-specific CD4+ T cells resulted in the control of infection.

We then investigated whether the cytotoxicity observed required cell-cell contact. We thus cultured and infected U373MG-CIITA in separate chambers using cell culture 3-μM diameter pore inserts. IE1-specific CD4+ T cell clone FzD11 was added only to the culture wells while inserts were devoid of CD4+ T cells. We observed, as shown in Fig. 6, that only U373MG-CIITA cells that had been in contact with the FzD11 T cell clone were subjected to cytotoxicity. Used as control, U373MG cells were not lysed by FzD11 T cell clone. Therefore, cell-cell contact was required for cytotoxicity.

Endogenous presentation of IE1 by infected U373MG-CIITA cells

Although Fig. 3 suggested that endogenous presentation could occur (phase 2 of presentation), it remained to be formally demonstrated. Therefore, we performed experiments to get rid of soluble IE1 that was responsible for phase 1 of presentation in Fig. 3. Total inoculum and supernatant from ultracentrifugation did contain IE1, as shown in Fig. 7, lanes 1 and 2, and as expected from results of CD4+ T cell clone activation shown in Fig. 3. Extensively washed and pelleted virus was shown to be completely devoid of soluble IE1 (lane 3). Successive washes show decreasing amounts of residual IE1 in supernatants (lanes 4–6). Nonspecific bands were consistently seen in crude inoculum and its supernatant (lanes 1 and 2).

Experiments were performed to determine whether soluble IE1-free CMV could induce CD4+ T lymphocyte activation. As shown in Fig. 8A, when soluble IE1-free CMV was used, IFN-γ production by FzD11 T cell clone increased from 133 to 762 pg/ml between 2 and 6 h postinfection. The kinetics of activation of FzD11 was different from that observed with crude inoculum: it showed a gradual increase over time of IFN-γ production, and no phase 1-type activation due to soluble Ag, contrary to that observed in Fig. 3 with untreated crude CMV. Moreover, kinetics of IE1 expression in U373MG-CIITA cells were compatible with kinetics of CD4+ T cell response and were confirmed using E13 mAb (Fig.
D) and IE1-specific rabbit anti-serum (data not shown). Those data suggested that endogenous presentation had occurred. To confirm that presentation of IE1 was due to endogenous protein produced by infected U373MG-CIITA, we compared the response of FzD11 to soluble IE1-free CMV, either infectious or inactivated by heat treatment. As shown in Fig. 8, soluble IE1-free (ultracentrifuged) virus did induce IFN-γ production and proliferation by FzD11, whereas heat-inactivated soluble IE1-free virus did not. These data strongly argue for endogenous presentation of IE1 by infected U373MG-CIITA, and excludes the possibility of a response to trace amounts of IE1. As a control, total CMV inoculum, whether heat-treated or not, induced activation of FzD11 thus demonstrating that heat treatment did not prevent exogenous presentation of IE1.

Control of CMV infection of U373MG-CIITA cells through endogenous presentation of IE1

Although we did show that endogenous presentation of IE1 occurred (Fig. 8) and that control of infection was specific (Fig. 5), it remained to be demonstrated that control of infection could be due to endogenous presentation of IE1. U373MG-CIITA cells were thus incubated with CMV ridden of soluble IE1 through several rounds of ultracentrifugation and washes. Fig. 9 shows that coinubcation of FzD11 with infected U373MG-CIITA cells

FIGURE 6. Importance of cell-cell contact in the cytotoxicity of anti-CMV CD4+ T cells. U373MG and U373MG-CIITA cells were cultured as schematically depicted either in the presence or the absence of IE1-specific CD4+ T cells. Cytotoxicity against infected APC was measured using a flow cytometry method based on the number of cells vs the calibrated number of fluorescent beads, as described in Materials and Methods.

FIGURE 7. Removal of soluble IE1 from CMV inoculum. CMV inoculum was ultracentrifuged, then washed by three additional ultracentrifugations in PBS. Supernatants and final pellets were analyzed by immunoprecipitation and Western blot for the presence of IE1.

FIGURE 8. Recognition of endogenous IE1 by CD4+ T cells. U373MG-CIITA cells were incubated with soluble IE1-free CMV (A) and the indicated preparations of CMV (B and C), and were fixed. IE1-specific FzD11 clone was added to the culture and proliferation and IFN-γ production were measured. Ultra, ultracentrifuged and washed; Inact., heat-inactivated.
markedly reduced virus production. This control of infection was specific because virus production by U373MG cells was not diminished by culture with FzD11. However, no cytotoxicity was observed (data not shown). Therefore, endogenous presentation of IE1 was capable of inducing recognition by CD4<sup>+</sup> T cells and control of infection.

### Discussion

We have shown, in the present study, that IE1-specific CD4<sup>+</sup> T cells recognize IE1 endogenously produced by the infected APC and that this recognition results in the control of infection in vitro.

We had previously shown that cytokines produced by IE1-specific CD4<sup>+</sup> T cells activated in the presence of APC and Ag can control infection of third-party permissive cells (19). However, activation resulted from recognition of synthetic peptide and recombiant protein by IE1-specific CD4<sup>+</sup> T cell clones. Therefore, we made the hypothesis that activation of IE1-specific CD4<sup>+</sup> T cells through presentation by MHC-II from infected APC could directly result in the control of infection. This has been confirmed in the present study. To reach this conclusion, we have constructed a cell model for Ag presentation by transfecting CIITA into CMV-permissive U373MG cells. Transfected cells were induced to express markers of the class II pathways and were potent APC that, upon infection, could present IE1 to specific CD4<sup>+</sup> T cell clones through the endogenous pathway.

The inhibition of MHCII expression previously reported by Tomazin et al. (40) did not appear, in our hands, to prevent CD4<sup>+</sup> T cell activation by infected APC. This may be due to the kinetics of expression of IE1 whose synthesis occurs earlier than the protein encoded by US2 which is responsible for the degradation of HLA-DR<sub>α</sub> and -DM<sub>α</sub> (40). However, the possibility that the consequences of MHCII molecules degradation by US2 appear only later in the infection needs to be investigated. Induction of decrease of MHCII expression and inhibition of CD4<sup>+</sup> T cell proliferation by CMV has also been described in macrophages (41), although the mechanisms are not yet elucidated. Nevertheless, our present data clearly demonstrate that IE1 is presented by infected APC to IE1-specific CD4<sup>+</sup> T cell clones in kinetics that are consistent with T cell activation and subsequent control of infection. The apparent discrepancy between numerous escape mechanisms and strong CD4<sup>+</sup> T cell response against CMV may reflect the host/virus balance.

U373MG-CIITA cells pulsed with either infectious or inactivated CMV induced a rapid, strong activation of IE1-specific CD4<sup>+</sup> T cells. The first peak of response was followed by dramatically decreased response after 16 h of incubation. Remarkably, a second peak of activation was observed only in the samples pulsed with active virus but not those pulsed with heat-inactivated virus. These kinetics presumably corresponded to 1) presentation of exogenous IE1 present in the inoculum whose presentation was diminished after 16 h of culture most likely due to exhaustion of HLA-DR/peptide complexes at the cell surface, and 2) endogenous presentation due to neosynthesized IE1. This conclusion was supported by the fact that supernatant from infected U373MG-CIITA cells did not induce CD4<sup>+</sup> T cell activation before day 5 of infection, presumably due to soluble IE1 released consequently to cytopathic effect of CMV. Virus inoculum did contain soluble IE1 as attested by phase 1 of activation and by the detection of IE1 protein in immunodetection assays. However, several rounds of washes provided us with active virus devoid of IE1. Therefore, the demonstration of endogenous presentation of nuclear protein IE1 relies on several arguments: 1) the trimodal curve of activation of CD4<sup>+</sup> T cell clone over time, 2) the kinetics of presentation of IE1 by APC infected with soluble IE1-free virus, and 3) the activation of CD4<sup>+</sup> T cells by soluble IE1-free virus that was lost upon heat treatment. Endogenous presentation of viral Ag may be of importance by focusing the CD4<sup>+</sup> T cell response to infected cells. It is obvious from our experiments that endogenous presentation was not the sole mechanism involved in activation of IE1-specific CD4<sup>+</sup> T cells. However, it appears that it was predominant within days 1 to 4 postinfection. Previous studies had demonstrated that cytokines such as IFN-γ and TNF-α can inhibit CMV infection, in the absence of cytotoxicity, when preincubated with cells before infection (19). Supernatant from IE1-specific CD4<sup>+</sup> T cell clones stimulated by infected U373MG-CIITA cells did contain IFN-γ that is likely to account in part for the decreased CMV Ag expression and inhibition of infection when preincubated with third-party cells before CMV infection (data not shown). Therefore, these cytokines may also be involved in the control of infection when no cytotoxicity is observed such as in the presence of lower concentrations of CD4<sup>+</sup> T cells (Fig. 4) and when endogenous presentation is involved from the start of CD4<sup>+</sup>/'APC interaction (Fig. 9).

The mechanisms involved in cytotoxicity mediated by CD4<sup>+</sup> T cell in response to exogenous IE1 required cell-cell contact. Anti-virus CD4<sup>+</sup> T cells have been shown to be able to kill infected targets using various pathways including Fas/Fas ligand, TNF-α, lymphotixin, TRAIL, TWEAK, and perforin/granzyme (42-45). We are examining whether different pathways of anti-CMV CD4<sup>+</sup> cytotoxicity require different levels of activation, as described (46, 47). This may explain the absence of cytotoxicity when endogenous presentation, possibly resulting in lower amounts of presented IE1, was involved. Alternatively, inhibitors of apoptosis encoded by CMV (48, 49) may prevent cytotoxicity in the context of the kinetics of endogenous presentation while readily available soluble IE1 in crude virus inoculum may induce CD4<sup>+</sup> T cell cytotoxicity before anti-apoptotic proteins are produced.

This is the first observation of specific recognition of CMV-infected cells by CD4<sup>+</sup> T cells. Recognition of infected monocytes by CD4<sup>+</sup> T lymphocytes was previously reported by Lindsley et al. (50). However it was unlikely to be targeted to neosynthesized CMV Ags, due to the absence of permissivity of monocytes to laboratory-adapted virus strains (28, 51). Recently, infection of permissive dendritic cells has been reported to induce T cell functional paralysis in a mouse model of CMV (30). In a human CMV model of infection, activated T lymphocytes were shown to be partially deleted, due to expression of apoptosis-inducing ligands
by infected dendritic cells (29). These data demonstrate that professional APC can be infected by CMV and show the strategies used by CMV to impair the initiation of T lymphocyte response. Our present model allows us to delineate the functional response of established CMV-specific CD4+ T lymphocytes to infection. Although the model of infected U373MG-CIITA and IE1-specific CD4+ T cells may not represent what happens when professional APC are infected, it allows us to evaluate the response to a specific Ag. More precisely, this paper shows that a CMV-encoded nuclear protein can be presented through an endogenous pathway. This is of importance because, while endogenous presentation of cytotoxic or transmembrane proteins has been widely reported (reviewed in Ref. 52), arguments for presentation of endogenous presentation of nuclear Ag have remained scarce. Endogenous presentation of nuclear protein EBV-encoded nuclear Ag 1 by MHC-II was reported recently (53), and hen egg lysozyme Ag targeted to the nucleus of murine sarcoma cells was shown to be presented by tumor cells to CD4+ T hybridoma cells (54). In a transgenic model, nucleustargeted β-galactosidase was able to induce tolerance to CD4+ T cells when expressed by thymic epithelial cells, but not bone marrow-derived APC (55). Our present study of response against IE1 is in agreement with these reports showing presentation of nuclear protein by MHC-II molecules. Furthermore, by using infectious virus CMV, our study further extends previous reports by showing functional consequences on infection of CD4+ T cell response to nuclear Ag.

Anti-IE1 specificity may be of importance because it is among the first proteins that are synthesized by the infected cells. Together with endogenous presentation to CD4+ T cells and focused response against infected APC, this may provide the CD4+ response against infected IE1 with an important role in vivo. Although our cell model may not be fully representative of professional APC such as macrophages and dendritic cells, we have shown in this study that U373MG-CIITA cells bear phenotypic properties that lead to effective Ag presentation, when compared with EBV-transformed B cells. This model may be of help to study both presentation of CMV Ags and escape from recognition by CD4+ T cells.

Although it is clear that the nuclear protein IE1 was presented through an endogenous pathway, it is thus far unclear in which cell compartment(s) Ag processing and encounter with HLA-DR occurred. In agreement with other reports (38, 39), staining for IE1 was exclusively nuclear in our confocal microscopy experiments. However, the extra-nuclear presence of IE1, although minor, has been reported in other studies (56, 57). The visualization of IE1 on membranes outside the nucleus using cell membrane fractionation (56) and electron microscopy (57) suggests that IE1 may be present in cell organelles. This localization needs to be examined by electron microscopy in our cell model, and it will be of interest to visualize colocalization with specific markers of Ag processing that may provide the functional basis for IE1 presentation by MHC-II. During reactivation, IE proteins are presumably produced first. Therefore, anti-IE1 specificity of T lymphocytes may be of particular interest to prevent extension of infection to other cells. The recognition of endogenous IE1 and subsequent release of IFN-γ, as described in the present study suggests that CD4+ T cells should be able to control CMV reactivation as well.

In conclusion, we have shown endogenous presentation of nuclear IE1 by CMV-infected APC to CD4+ T cells. This presentation results in CD4+ T cell activation evidenced by proliferation and production of IFN-γ and control of infected cells. Our observations may implicate IE1-specific CD4+ T cells in the recognition and control of CMV-infected APC.

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References


