An Endogenous Processing Pathway in Vaccinia Virus-infected Cells for Presentation of Cytoplasmic Antigens to Class II-restricted T Cells

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Summary

The recognition of virus-infected cells by class I MHC-restricted cytotoxic T cells requires endogenous processing of antigen for presentation. It is still unclear whether endogenous processing of antigen can be utilized by class II MHC molecules for presentation. To test this possibility, a human B cell line expressing HLA-A2 and HLA-DR1 was infected with a recombinant vaccinia virus expressing the Influenza A virus M1 matrix protein (VAC-M1) and was assayed for lysis by different M1-specific cytolytic T cell lines, restricted by either HLA-A2 or by HLA-DR1. Class II-restricted lysis of VAC-M1-infected cells did occur. This lysis required de novo M1 synthesis and was not due to exogenous antigen. Several properties of the endogenous processing pathway for class II-restricted presentation were different from those of the pathway utilized by class I molecules. First, class II-mediated recognition of VAC-M1 infected cells was less efficient, requiring higher doses of virus and longer infection times, than the class I-mediated recognition. Second, chloroquine completely blocked presentation of endogenous M1 to class II-restricted T cells but had no effect on the class I-restricted presentation. Third, the class II-restricted presentation of M1 was only mildly affected by Brefeldin A, a drug that prevents transport from the endoplasmic reticulum to the Golgi, whereas the class I-restricted presentation of M1 was completely abrogated by this drug. These data demonstrate the existence of an endogenous processing pathway for the presentation of cytosolic antigen by class II molecules and show that this pathway is distinct from the one used for presentation by class I molecules.

Antigen processing refers to the multiple biochemical and cellular events that lead to the formation of an immunogenic complex between a foreign antigen and an MHC molecule of the presenting cell, such that this complex can be recognized by a T cell. The processing requirements for presentation by class I or by class II MHC molecules differ. Exogenous antigen, in the form of inactivated virus or soluble protein, can be efficiently processed by antigen presenting cells, such as B cells and macrophages, for presentation by class II molecules (1). In contrast, class I molecules generally do not present exogenous protein antigen (2, 3), at least not in vitro (4). The exogenous processing pathway involves antigen uptake by the presenting cell and delivery of antigen into an acidic intracellular compartment where processed fragments may bind to newly synthesized class II molecules on their way to the cell surface (5, 6). Class II molecules internalized from the cell surface may also contribute to the presentation of exogenous antigen by murine B cells (7, 8). The exogenous processing pathway can be blocked by agents, such as chloroquine, that prevent acidification of intracellular organelles.

The main requirement for presentation by class I molecules seems to be the presence of antigen in the cytosol (2, 9). Peptide fragments, presumably derived from cytoplasmic degradation of the antigen, may translocate into the endoplasmic reticulum where they bind to newly synthesized class I molecules (10, 11). Peptide fragments synthesized directly in the cytosol from mini-gene constructs inserted into recombinant vaccinia viruses were efficiently presented by class I molecules (12–14) but not by class II molecules (14). The inability of class II molecules to present endogenously synthesized transmembrane hemagglutinin of influenza virus (3) led to the proposal that class II-restricted presentation was limited exclusively to exogenous antigens (15, 16).

However, an obvious pathway for the processing of endogenous cell surface antigens for class II-mediated presentation would be internalization into an endosomal compartment. Several studies on the presentation of membrane-associated antigens are compatible with this possibility (17–22). Although internalization of endogenously synthesized surface antigen for class II-mediated presentation would represent a pathway similar to uptake of exogenous antigen, it is notably different in that it would enable the immune system to encounter cell-specific self antigen in the context of class II molecules. Be-
cause the presentation of internal antigens by class II molecules could potentially affect many immune functions, such as selection of T cells in the thymus, peripheral T cell tolerance, autoimmunity, alloreactivity, and the generation of help after viral infections, it is important to determine whether cellular mechanisms exist that lead to class II–restricted presentation of endogenous cytoplasmic antigens.

Whether cytosolic antigen can be processed endogenously for class II–restricted presentation is still debated. Hepatitis B surface antigen synthesized endogenously from a recombinant vaccinia virus was processed for class II–restricted presentation, as measured in a 4-d proliferation assay (23). This long infection time and the unusual membrane association of this antigen make it difficult to distinguish between internalization and cytosolic processing as potential mechanisms for presentation. It remains important to test whether such endogenous processing for class II–restricted presentation is applicable to cytosolic antigens. Murine L cell fibroblasts transfected with measles virus genes encoding cytosolic proteins did process these antigens endogenously for presentation to CD4+ T cells (24). However, the lack of human invariant chain in those transfected cells may account for their ability to utilize an endogenous processing pathway (25). Another study, using cells incubated with influenza virus at an acidic pH, and treated simultaneously with chloroquine and Brefeldin A, concluded that class II molecules can utilize the endogenous cytoplasmic pathway used by class I molecules (26). This conclusion is difficult to reconcile with the evidence that a pre-processed cytoplasmic peptide containing epitopes for both class I– and class II–restricted T cells, expressed from a recombinant vaccinia virus, was presented by class I but not by class II molecules (14), unless the ad hoc assumption is made that vaccinia virus infection selectively inhibits the transport of class II molecules.

In this study, a recombinant vaccinia virus encoding the M1 matrix protein of influenza A virus (VAC-M1)1 was used to test whether cytosolic processing would lead to presentation of a HLA-DR1-restricted M1 epitope, in addition to a HLA-A2-restricted epitope. Direct evidence is provided that endogenously processed M1 protein can be presented to class II–restricted cytolytic T cells. Furthermore, kinetic and pharmacological properties of the M1 processing for class II–mediated presentation suggest that distinct endogenous pathways are used specifically for presentation by either one or the other class of MHC molecules.

Materials and Methods

Cells. The hemizygous HLA-A2,DR1 EBV-transformed cell line 45.1 (27) was used as target cell in all experiments. Cells were maintained in RPMI 1640 (Hazeltone Biologics Inc., Lenexa, KS) supplemented with 2 mM glutamine, 10 μg/ml gentamicin and 10% heat-inactivated FCS.

T Cell Lines. T cell lines 109.2B2 and 130AC6 are HLA-DR1-restricted and recognize influenza A M1 matrix derived peptide 1-26 (28). HLA-A2-restricted T cell line 161 recognizes the M1 peptide 56-68 (28). All lines were generated in limiting dilution at 10 cell/well by direct stimulation with peptide and were kindly provided by W. Biddison (Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, NIH). T cell lines were grown in Iscove’s modified Eagle’s medium supplemented with 10% heat-inactivated human A-serum, 10 μg/ml gentamicin, and 10 U/ml recombinant IL-2 (kindly provided by the Cetus Corp., Emeryville, CA). The cells were maintained by weekly stimulation with irradiated (8,000 rad) feeder cells consisting of a 10:1 mixture of mononuclear cells and 45.1 cells treated with 10 μg/ml of the corresponding peptide for 2–3 h at 37°C, washed, and resuspended in the same medium.

Antigens. Purified Influenza A M1 matrix was a gift of J. Yewdell (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, NIH). M1 peptides 17-31 and 56-68 were kindly provided by J. Rothbard (Imperial Cancer Research Fund, London, UK) and W. Biddison, respectively.

Virus. Influenza A/Sichuan/21/87 virus was obtained from M. Williams (Center for Biologic Evaluation and Research, Food and Drug Administration, Bethesda, MD) as infectious allantoic fluid. Purified (1.6 × 109 PFU/ml) recombinant vaccinia virus containing the influenza M1 gene (VAC-M1) has been described (29) and was kindly provided by Dr. B. Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, NIH). UV-inactivation of influenza or vaccinia virus was achieved by a 15-min exposure to 1,200 μW/cm2 of a short-wave UV light.

Target Sensitization. For peptide sensitization, 5 × 106 45.1 cells were cultured with 10 μg/ml peptide in RPMI 1640 with 5% FCS for 1 h, unless otherwise indicated, at 37°C. The same was done for sensitization with M1 protein at different concentrations. For infections with recombinant vaccinia virus, 5 × 107 Cr-labeled 45.1 cells were resuspended in 500 μl RPMI 1640 with 5% FCS containing different doses (25, 50, or 100 PFU/cell) of purified virus. After 1 h on a rotator at 37°C, 1 ml medium was added and the cells were kept under continuous rotation for a further 0, 1, or 4 h, for a total of 1, 2, or 5 h of infection. Cells were then washed, counted, and used as targets for CTL assays. For experiments requiring chloroquine treatment, 5 Cr-labeled target cells were washed and preincubated for 10 min in medium containing 80 μM chloroquine (Sigma Chemical Co., St. Louis, MO) before sensitization. The same concentration of chloroquine was maintained throughout sensitization (including washes), but it was reduced to 10 μM for the CTL assay. Anisomycin (Sigma) and Brefeldin A (kindly provided by R. Klausner, Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, NIH) were used at 25 μg/ml and 0.5 μg/ml, respectively, at the time of sensitization, and were maintained at the same concentration during the CTL assays.

Cytotoxicity Assays. Cytolytic activity was measured by standard 4-h 51Cr-release assays. Target cells were labeled with 100 μCi 51Cr for 1 h at 37°C and washed in RPMI 1640 with 5% FCS. After the first wash, targets were sensitized or infected, washed again, counted, and resuspended in the same medium at a concentration of 40,000 cells/ml. 2,000 targets per well (50 μl) were mixed with 100 μl effector cells at different ratios in V-bottomed microtiter plates (Nunc, Roskilde, Denmark). Supernatants were harvested and counted. Percent specific 51Cr-release was calculated as:

\[ \text{specific } 51\text{Cr-release} = \frac{(\text{release by CTL} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100 \]

All points were mean cpm from duplicate experiments against triplicate controls for maximum and spontaneous release. Maximum and spontaneous releases were defined as cpm released from targets incubated with 100 μl 0.1M HCl or

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1 Abbreviation used in this paper: VAC-M1, recombinant vaccinia virus encoding the influenza A virus M1 matrix protein.
100 µl medium, respectively. Spontaneous release ranged between 15–30% of the maximum release in all experiments.

**Cell Mixing Experiments.** In a typical experiment with VAC-M1, infection of 3Cr-labeled cells starts with a 1-h incubation with concentrated virus to allow entry, followed by a dilution and further incubation for 4 h to allow infection to proceed, and by a 4-h CTL assay during which infection of the target cells continues. Even though processing of exogenous M1 can take place in only 1 h, a rigorous mixing experiment was designed in which uninfected 3Cr-labeled cells were mixed with VAC-M1-infected cells for 4 h before and during the 4 h CTL assay. To reduce the possibility of virus spreading to uninfected cells during the coculture, cells infected with VAC-M1 for 2 h were washed before mixing. To control that a wash 2 h after addition of VAC-M1 did not reduce the efficiency of VAC-M1 infection, and that mixing per se did not reduce lysis by CTL, a reverse mixing experiment with 3Cr-labeled infected cells and uninfected uninfected cells was carried out. After the 2 h of infection cells were washed and counted. 200,000 cells were mixed with equal numbers of 3Cr-labeled uninfected 45.1 cells and incubated for 4 h at 37°C in continuous rotation. Cells were then washed and resuspended in RPMI 1640 with 5% FCS to a concentration of 80,000 cells/ml. 50 µl of this mixture (containing 2,000 cells of each type) were then used as targets for CTL assays.

**Results**

**Specificity of the Cytolytic T Cell Lines.** T cell lines used here were specific for the HLA-A2-restricted epitope of the Influenza virus M1 antigen, or the HLA-DR1-restricted M1 epitope. The B cell line 45.1 expressing A2 and DR1 was chosen as target for CTL assays. The T cell line 161 is specific for the M1 peptide 56-68 in the context of A2, and T cell lines 109.2B2 and 130.1C6 recognize the M1 peptide 17-31 presented by DR1 (Fig. 1). In addition, the DR1-restricted T cell lines 109.2B2 and 130.1C6 lysed targets treated with the purified M1 protein. Fig. 2a shows results obtained with the line 130.1C6. Recognition of target cells by the DR1-restricted lines required three orders of magnitude less M1 protein than M1 peptide 17-31, on a molar basis. Addition of chloroquine to the target cells during sensitization with M1 protein inhibited the cytolyis; complete inhibition re-quired 80 µM chloroquine (Fig. 2b). Whereas processing of inactivated influenza virus for presentation was also chloroquine-sensitive (data not shown), presentation of the M1 peptide 17-31 was not inhibited (Fig. 2b).

**Target Cells Infected with VAC-M1 Are Lysed by Both Class I-and Class II-restricted M1-specific T Cell Lines.** 45.1 cells were infected for 5 h with increasing doses of VAC-M1 and tested for lysis by the A2- or the DR1-restricted M1-specific lines (Fig. 3a). Class I-mediated lysis was more efficient: at 25 PFU/cell it exceeded the class II–mediated lysis obtained at 100 PFU/cell. 45.1 cells were infected with 100 PFU/cell for increasing times and tested for lysis (Fig. 3b). Whereas M1 presentation by A2 occurred after only 1 h of infection, presentation by DR1 was barely detectable during the first 2 h of infection. This experiment detected true differences in the kinetics of processing and presentation of M1, rather than differences in infection and M1 synthesis, because the
same infected targets were assayed in parallel with either the class I- or the class II-restricted cytolytic T cells.

As a specificity control, 45.1 cells infected with a recombinant vaccinia virus encoding the influenza A H3 hemagglutinin, and expressing surface H3, were not susceptible to lysis by either 109.2B2 or 130.1C6 T cell lines (data not shown).

Lysis of VAC-M1-infected Targets by Class II-restricted Cell Lines Requires M1 Synthesis. Two control experiments were carried out to rule out the possibility that the VAC-M1 preparation was contaminated with M1 protein at a level sufficient to result in exogenous processing for class II-restricted presentation. Despite the use of gradient-purified VAC-M1 these controls were important because, as shown in Fig. 2 a, incubation with as little as 0.03 μg/ml (1 nM) M1 protein for 1 h leads to presentation. First, when the VAC-M1 preparation was inactivated with UV light, presentation was abolished (Fig. 4 a). UV light does not destroy the M1 epitope because UV-inactivated influenza virus can be presented (Fig. 4 b). Second, treatment of infected cells with the protein synthesis inhibitor anisomycin also abolished M1 presentation (Fig. 4 c). This demonstrates the requirement for M1 synthesis because anisomycin only inhibited partially the processing of exogenous UV-inactivated influenza virus (Fig. 4 d). This inhibition occurred after 5 h of treatment and was probably due to depletion of class II molecules by protein synthesis inhibition.

The Processing of M1 in VAC-M1-infected Cells for Class II-restricted Presentation Is Endogenous. Rigorous mixing experiments were carried out to test the possibility that M1 protein accumulated in the medium of VAC-M1-infected cells, thus providing an exogenous source of antigen for processing and presentation. Uninfected 51Cr-labeled 45.1 cells were mixed with VAC-M1 infected cells for 4 h before and during the 4-h CTL assay. Fig. 5 shows that (a) the mixing protocol did not reduce the level of lysis of infected cells, as compared with the usual protocol without cell mixing, and (b) lysis of infected cells cannot be accounted for by the presence of exogenous M1 because uninfected cells were either not lysed, or barely so, in the CTL assay. In agreement with the evidence that neither VAC-M1 nor M1 protein was transferred to uninfected cells, lysis of uninfected cells by class I-restricted CTL did not occur after mixing with infected cells (not shown).

The Endogenous Processing Pathway for Class II-restricted M1 Presentation Is Different from the One Used for Class I-restricted Presentation. Class I-restricted antigen presentation appears to require the acquisition of peptide by a newly synthesized class I molecule in the endoplasmic reticulum (10, 11). Consistent with this view, processing for class I-restricted presentation is not inhibited by chloroquine (3) but it is completely abolished by Brefeldin A (30, 31), a drug that prevents export of proteins from the endoplasmic reticulum. To test whether class II molecules might share the endogenous processing pathway utilized by class I molecules, inhibition experiments with chloroquine and Brefeldin A were carried out. Chloroquine was used, under conditions that totally block presentation of exogenous purified M1 protein, with VAC-M1-infected cells to test its effect on endogenously processed M1. The same infected cells were assayed in parallel with either the class I-restricted or the class II-restricted T cell lines (Fig. 6). The class I-mediated T cell recognition was unaffected by treatment with chloroquine. This result shows that infection with vaccinia virus and expression of the M1 protein
took place in the presence of chloroquine and, as expected, that cytoplasmic processing of M1 and its presentation by class I were not inhibited by this drug. However, the class II–mediated response was totally abrogated. This inhibition can be interpreted in two ways. First, endogenous processing for class II–mediated presentation may involve a chloroquine-sensitive intracellular compartment. Second, the chloroquine inhibition may not be at the level of processing but may be mediated by the delayed dissociation of the invariant chain from the αβ dimers (32, 33). If endogenously processed antigen interacts with class II molecules in the endoplasmic reticulum, class II–restricted presentation should be inhibited by Brefeldin A. Target cells were treated with Brefeldin A and infected with VAC-M1 (Fig. 7). This 5-h treatment resulted in a complete inhibition of the class I–mediated presentation. In contrast, a mild inhibition of the class II–mediated presentation was noted. Therefore, transport of class II molecules out of the endoplasmic reticulum is not required for presentation of endogenous antigen.

**Discussion**

The possibility that endogenous processing of cytosolic antigen could lead to presentation by class II molecules was investigated using a well-defined antigenic system. The M1 matrix protein of influenza A virus is a cytosolic antigen with defined epitopes recognized by T cells restricted by either HLA-A2 or HLA-DR1. Human cytolytic T cell lines specific for the DR1-restricted M1 epitope, as well as a T cell line specific for the A2-restricted M1 epitope, were used to assay presentation by a HLA-A2,DR1 B-cell line. A recombinant vaccinia virus encoding the M1 protein was used to obtain endogenous M1 synthesis in infected cells, in the absence of an exogenous source of M1 protein. The influenza virus M1 protein was well suited for this study because it is known to remain in the cytosol and the nucleus of infected cells (34) and to remain in the cytosol of VAC-M1 infected cells, even after 14 h of infection (29). As expected, the 45.1 B cell line used here did not express detectable surface M1 after 5 h of infection with VAC-M1 (data not shown).

Unequivocal evidence was obtained that endogenously synthesized M1 protein can be processed and presented by class II molecules. A purified recombinant vaccinia virus, free of M1 protein, was used to infect target cells under conditions that did not lead to detectable accumulation of exogenous M1 protein. Such infected target cells were lysed by both class I– and class II–restricted T cells, specific for distinct M1 epitopes. The demonstration of an endogenous processing pathway for presentation of cytoplasmic proteins by class II molecules was thus derived without relying on assumptions about the specificity of certain drugs that interfere with cellular functions.

The evidence that a viral cytosolic antigen can be presented by class II molecules suggests that endogenous self proteins may also be processed and presented to CD4+ T cells. Contrary to a situation where only exogenous antigen can be presented, this would provide the possibility of cell-specific antigen presented in the context of class II molecules. Class II–positive thymic epithelial cells that are responsible for the positive selection of CD4+ T cells (35) may indeed, as was suggested (36), utilize cell-specific peptides to select T cells, some of which will escape the negative selection mediated by class II–positive bone marrow–derived dendritic cells in thymic medulla. Cell-specific presentation of endogenous peptides could also explain several observations on alloreactive class II–restricted T cells. Certain alloreactive CD4+ T cells
recognized the allo-class II molecule only when expressed on certain cells, e.g., on B cells but not on macrophages (37); again it was suggested that it was probably due to the recognition of a cell-specific peptide presented by the class II molecules. Such a cell-specific peptide need not be derived from re-uptake of exogenous antigen but may well result from endogenously antigen processing. The range of self peptides that can potentially bind to class II molecules is thus vastly increased. The need to develop tolerance to all endogenously derived peptides presented at a sufficiently high level places an additional burden on the immune system. Endogenously derived peptides also provide potential targets for autoimmune reactions. On the other hand, presentation of endogenous antigen has the obvious advantage that T cell help would be initiated rapidly after infection of class II-positive cells.

Endogenous M1 was less efficiently processed for class II-associated recognition than for class I-associated recognition, both in the dose of recombinant vaccinia virus required and in the kinetics of processing and presentation. These differences reflect either distinct processing pathways, operating separately for presentation by the two classes of MHC molecules, or, in the case of a shared pathway, a lower efficiency in the transport of class II molecules to the cell surface, or in the processing or the binding of the DR1-restricted epitope. In contrast to the exogenous-endocytic processing for class II-mediated presentation, cytosolic processing for class I-mediated presentation is insensitive to chloroquine (3). Treatment with chloroquine, at a dose that totally abolished processing of exogenous M1 protein, before and during VAC-M1 infection, had no effect on the A2-restricted recognition of infected cells but blocked efficiently the DR1-restricted recognition by M1-specific CTL lines. This result is consistent with the existence of separate processing pathways for the two classes of MHC molecules. However, interpretation of the chloroquine effect on class II-mediated presentation is complicated by the fact that the invariant chain, associated intracellularly with the class II α/β dimer until their delivery into a post-Golgi acidic compartment (33), is dissociated from the α/β dimers at a slower rate in the presence of chloroquine (32).

The most direct evidence for the existence of separate pathways for processing of endogenous antigens was obtained with Brefeldin A. This drug causes a rapid redistribution of the Golgi compartment to the endoplasmic reticulum, thus preventing export of newly synthesized proteins from the endoplasmic reticulum (38-40). Whereas the A2-restricted recognition of VAC-M1-infected cells was totally abolished by treatment with Brefeldin A, this drug caused only a modest inhibition of the DR1-restricted recognition. Therefore, peptide loading of endogenously processed antigen onto class II molecules does not take place in the endoplasmic reticulum, implying separate processing pathways for the two classes of MHC molecules. Under certain conditions, Brefeldin A can inhibit presentation of exogenous antigen by class II molecules (41). This inhibition was similar to that observed in cells treated with cycloheximide, indicating a need for newly synthesized class II molecules in the presentation of endogenous antigen. In the present study, a 5-h treatment with Brefeldin A of the target cells infected with a recombinant vaccinia virus was obviously insufficient to deplete the internal pool of class II molecules. A more pronounced inhibition by Brefeldin A of class II-restricted presentation was observed by others (26) in cells infected for 5 h with influenza virus. It is conceivable that the kinetics of class II molecule transport from the endoplasmic reticulum to the cell surface is affected differentially by these two viruses.

The evidence that class II-mediated presentation of endogenously processed antigen is not dependent on loading of peptides in the endoplasmic reticulum is consistent with the finding of Sweetser et al. (14) that a pre-processed cytoplasmic peptide containing overlapping epitopes for class I- and class II-restricted T cells was presented by the class I but not by the class II molecules. One possible explanation for the inability of class II molecules to utilize the class I pathway of presentation is that the invariant chain associated with newly synthesized class II molecules may prevent peptide binding (25, 42). Peptide loading onto class II molecules would occur only in a post-Golgi compartment, where the invariant chain is proteolytically cleaved from the α/β dimers (33). This model does not rule out the possibility that certain peptides with high affinity for the class II molecule may be able to bind in the presence of the invariant chain. One distinction between the endogenous pathways for the two classes of MHC molecules may be that cytosolic peptides derived from degraded proteins translocate into the endoplasmic reticulum but not into the compartment for class II-mediated presentation, whereas certain cytosolic proteins translocate into the latter compartment.

The processing pathway for presentation of cytosolic proteins by class II molecules must involve some targeting mechanism that translocates these proteins into the compartment where processing for class II-mediated presentation takes place, presumably a post-Golgi compartment. The current evidence cannot distinguish whether cytosolic proteins destined to this compartment must translocate first to the cell surface for re-internalization or can be translocated directly into the proper intracellular vesicle. In either case, a specific mechanism is required that will target cytosolic proteins to the proper destination, and the immunological implications of class II-restricted presentation of endogenous antigen remain unchanged. Translocation of cytosolic proteins into lysosomes (43) and autophagy (44) are both mechanisms that could be utilized for class II-mediated presentation. Although lysosomes are generally regarded as a terminal destination, it remains to be established whether peptides generated by lysosomal degradation can bind to functional class II molecules. Alternatively, translocation of cytosolic proteins directly into endosomes may also occur.
References