

CLASS II MAJOR HISTOCOMPATIBILITY
COMPLEX-RESTRICTED T CELLS SPECIFIC FOR A
VIRION STRUCTURAL PROTEIN THAT DO NOT
RECOGNIZE EXOGENOUS INFLUENZA VIRUS

Evidence that Presentation of Labile T Cell Determinants Is
Favored by Endogenous Antigen Synthesis

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Viral protein synthesis within host cells exerts a major influence upon T cell responses to influenza virus (1-4). In general, class I MHC-restricted recognition is favored by occurrence of viral biosynthesis while class II-restricted responses to the same protein can be elicited by inert virions. The composition of T cell populations can be influenced by the infectivity of the viral antigen. In a recent study, Morrison et al. (5) observed that *in vitro* restimulation of virus-primed murine splenocytes with infectious virus yielded mainly class I MHC-restricted CTL, while noninfectious virus under the same conditions induced primarily class II-restricted CTL. Antigen processing appears to be required for presentation of many class I-(6-8) and class II-(9-11) restricted influenza virus T cell determinants, although exceptions are known (12, 13). Cellular events associated with viral protein biosynthesis may favor presentation with class I molecules (reviewed in references 14 and 15). For example, Braciale and coworkers (16), using a vaccinia virus recombinant bearing the hemagglutinin (HA)¹ gene of influenza virus, studied antigen recognition requirements for class I- and class II-restricted CTL clones. Although influenza HA was absent on input vaccinia, synthesis of HA within target cells permitted lysis by class I-restricted T cells while failing, during the same time interval, to sensitize the cells for class II-mediated lysis. Although endogenous synthesis is evidently the major introduction route of viral proteins for class I-restricted processing, viral replication requirements were able to be bypassed by pretreatment of influenza virions to inactivate their receptor-destroying enzyme activity (17), although virus uptake and fusion with cellular membranes was required for formation of class I target structures (8). This appears to be an alternate means of engaging class I-associated processing. On the other hand, class II-restricted responses reported so far showed no such special requirements for utilization of antigens present on virus particles.

We now report evidence that certain class II MHC restricted T cell responses to influenza virus structural proteins are strongly dependent upon viral infectivity.

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¹ *Abbreviations used in this paper:* HA, hemagglutinin; HAU, hemagglutinin units; NA, neuraminidase.

T cell recognition of a determinant of the influenza neuraminidase (NA) envelope glycoprotein was blocked by preventing viral biosynthesis. Although shown to be a linear determinant restricted to I-E^d, the replication requirement is typical of a class I-restricted site. Studies of two I-E^d-restricted determinants of the HA molecule revealed that, although both can be generated from replication-inactive exogenous virus, one determinant showed a 10-fold increase in expression in presence of infectious virus while the other was only slightly enhanced. This enhancement of presentation by infectious virus appears to depend upon individual properties of T cell sites; data suggest a correlation to the lability of the determinant.

Materials and Methods

Viruses and Antigens. Influenza virus A/Puerto Rico/8/34 (PR8; H1N1) was grown in the allantoic cavity of embryonated hen eggs and purified by sedimentation and banding on sucrose density gradients. Virus concentration in hemagglutinating units (HAU) was determined by chicken red cell agglutination (18). Inactivation of virus replication was achieved by exposure to short-wave UV light using a UVGLD58 Mineralight lamp (Ultraviolet Products, San Gabriel, CA) at 240 $\mu\text{W}/\text{cm}^2$ for 15 min. Staining for viral antigens of Madin Darby Canine Kidney cells exposed to dilutions of treated virus confirmed the effectiveness of the inactivation. The vaccinia recombinant virus used to screen T cell hybridomas for neuraminidase specificity was generated by Dr. G. Smith (19) and was the generous gift of Dr. J. Yewdell, National Institutes of Health (Rockville, MD). Purified HA/NA rosettes were prepared by ammonium deoxycholate treatment of purified virus after the method of Laver and Webster (20). Purified bromelain-solubilized HA was prepared according to Brand and Skehel (21). HA-free NA was purified from PR8 virus by the method of Gallagher et al. (22). Yield of highly purified NA was very low and its use was restricted to quantitative immunoassays. Protein concentrations of pure HA and NA were determined by quantitative amino acid analysis.

Antibodies. The PR8 HA-specific mAb Y8-1C1 was previously described (23) and the type B/Lee HA-specific mAb 1.3C9.4 were both kindly provided by Drs. Walter Gerhard and Peggy Scherle, Wistar Institute, Philadelphia, PA. The monoclonal anti-neuraminidase antibody NA2-1C1 was the generous gift of Dr. J. Yewdell, National Institutes of Health, Rockville, MD. All of these antibodies (in the form of culture fluid) were of the IgG1 isotype. Monoclonal rat anti-mouse IgG1 was produced at the Wistar Institute by Dr. Louis Staudt. The peroxidase conjugated mouse anti-rat mAb was purchased from Accurate Chemical & Scientific Corp., Westbury, NY.

Cell Lines. The B cell lymphoma A20-1.11 (A20), a cloned APC, originally isolated and described by Kim et al. (24), was the generous gift of J. Kappler (National Jewish Hospital, Denver, CO). The protein synthesis inhibitors cycloheximide and emetine (Sigma Chemical Co., St. Louis, MO) inhibited amino acid incorporation in these cells when used at indicated concentrations. T cell hybridomas specific for two I-E^d-restricted determinants of PR8 HA at residues 111-119 (site 1) and 302-313 (site 3) have been described (25-27).

The neuraminidase-specific T cell hybridoma NA-2.10 was generated by immunization of BALC/c mice with PR8 virus as previously described (25) and screening of fused cells with the NA-bearing recombinant vaccinia virus. The precise antigenic region of NA recognized by these cells is not known.

All cells were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 5% (vol/vol) FCS.

Fixation of APC. The method of Allen and Unanue (28) using freshly-made 0.5% (wt/vol) paraformaldehyde was used with the modifications that have been described previously (11).

T Cell Stimulation Assay. The ability of APC to stimulate influenza-specific T hybridomas was tested as previously described (11). To isolate the effect of active viral protein synthesis from potentially complicating factors, such as infection of T cells or toxic effects of the virus, A20 cells used as APC were pulsed briefly with influenza virus, fixed with paraformaldehyde after various culture periods, and washed extensively before addition to T cell stimulation

assays. The fixed A20 cells retained ability to be recognized by T cells and could be assayed for expression of viral determinants. Briefly, APC that had been previously exposed to antigen and were paraformaldehyde fixed were diluted two- or threefold in 96-well microtiter plates from starting concentrations of 4×10^5 cells/well. Fixed APC or untreated APC (used at 5×10^4 cells/well) plus antigen (for unpulsed APC) and T hybridomas (3×10^4 cells/well) were co-incubated in serum-free IMDM at $37^\circ\text{C}/6\% \text{CO}_2$ for 48 h. Culture supernatants were assayed for IL-2 produced by stimulated T cells using the IL-2-dependent indicator cell line CTLL (29). Viability of CTLL after co-incubation with the supernatants for 24–36 h was quantitated by a modification of the original method of Mosmann (30) using cleavage of the dye (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (31, 32). Color development was measured using a platereader (model EL309; Bio-Tek Instruments, Inc., Burlington, VT) at 570 nm (750 nm reference wavelength).

Assay for Total Cellular HA and NA by ELISA. APC that had been pulsed with virus were harvested by centrifugation, washed twice with PBS, and suspended at 10^7 cells/ml in ice-cold 0.15 M sodium bicarbonate/carbonate buffer, pH 9.3, and 2 mM phenylmethylsulfonyl fluoride. The cell suspension was then micro-tip probe sonicated at ~ 80 watts (L Converter probe sonicator; Bronson Sonic Power Co., Danbury, CN) on ice for 3 min. Lysates (25- μl volumes) were then diluted into Costar Serocluster half-area EIA plates in twofold steps in medium containing lysates of A20 cells (unpulsed cells lysed at the same density). Purified PR8 virus and samples of purified HA and NA of known concentration were diluted in parallel. Wells were dried at room temperature followed by treatment with 0.5% gelatin in 0.15 M borate-buffered saline, pH 8.3. The wells were then tested for presence of HA and NA by ELISA using mAbs (see above) specific for PR8 HA, NA, or, for negative controls, B/Lee HA, followed by rat anti-IgG1 antibody and peroxidase-conjugated mouse anti-rat antibody. The reactions were developed using the TMB Microwell Peroxidase Substrate Kit, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, and absorbancies were measured at 450 nm as described above.

Results

Replicative Virus Is Required for Expression of the NA Determinant upon Antigen-pulsed APC. A20 cells were pulsed with the same concentration of PR8 influenza virus which was either (a) infectious, (b) UV-inactivated, or (c) infectious, but in presence of the protein synthesis inhibitors cycloheximide or emetine. After 24 h in culture, pulsed APC were paraformaldehyde fixed and placed in T cell assays with several influenza-specific T hybridomas. Each of the T cells showed similar sensitivities to antigen by IL-2 release in response to the same APC population (Fig. 1). Fig. 1 a shows the response of a T hybridoma recognizing a determinant of the NA. Most notable is the complete absence of stimulation where virus had been rendered incapable of replicating its proteins within host cells either by UV or by presence of protein synthesis inhibitors. The responses of two I-E^d-restricted T cells recognizing influenza HA were tested upon the same APC groups. T cells recognizing sites in the HA at amino acids 111–119 (site 1) and 302–313 (site 3) of the HA1 polypeptide (25, 33) are able to be stimulated by APC pretreated with either inactivated or infectious virus (Fig. 1 b and c). However, APC pulsed with infectious virus showed a greater stimulatory potency for both of the HA-specific T cells. The enhancement of stimulation was nearly 10-fold for site 3-specific cells (Fig. 1 c) as opposed to a slight increase observed for site 1 expression (Fig. 1 b). Since identical results are obtained whether virus was inactivated by UV or by affecting protein synthesis within the host cell, differences in T cell stimulation appear to be based upon presence or absence of newly synthesized viral proteins within APC.

The Appearance of the NA T Cell Determinant Follows Closely the Synthesis of NA Protein

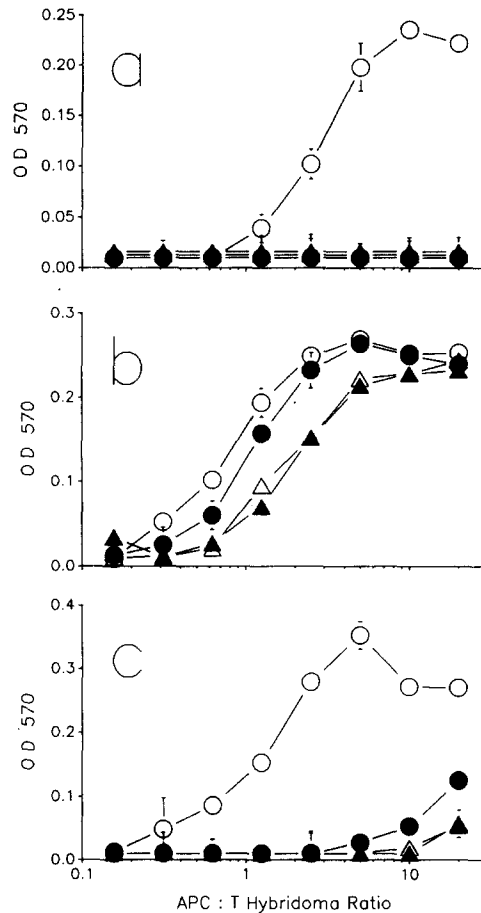


FIGURE 1. Effect of inhibition of viral protein synthesis upon presentation of Th determinants. APC were pulsed with infectious or UV-inactivated influenza PR8 virus and incubated 24 h before paraformaldehyde fixation. Cells pulsed with infectious virus were incubated with normal medium or medium containing either 20 $\mu\text{g}/\text{ml}$ cycloheximide or 10 μM emetine. The fixed APC were tested for expression of Th determinants in T cell stimulation assays with the HA- and NA-specific T hybridoma clones NA-2.10 (a, NA specific); 3.2B11 (b, HA/site 1 specific); and 2LD3-11 (c HA/site 3 specific). APC were pulsed with UV-treated virus (●), or infectious virus in presence of medium only (O), cycloheximide (Δ), or emetine (▲).

within Infected Cells. To determine the relationship between viral protein synthesis and T cell determinant generation, the following timing experiment was performed. APC were pulsed with infectious or UV-inactivated virus and harvested after intervals of incubation at 37°C. Aliquots at each time point were either: (a) lysed by sonication and analyzed for the levels of total NA and HA by ELISA, or (b) fixed with paraformaldehyde and placed in T cell stimulation assays to determine the relative amounts of the three determinants expressed at the APC surface. Fig. 2 a shows that newly synthesized NA appears between 4 and 8 h post-infection. FACS analysis also showed the onset of NA surface expression to occur in the same interval (data not shown). The appearance of the T cell determinant, as indicated by ability of the APC to stimulate the NA-specific T hybrid, occurs between 8 and 12 h post-infection. Thus, the expression of the NA T cell determinant follows the appearance of the newly synthesized NA. This is contrasted with the onset of Th determinant expression for HA site 1. In this case, ability to recognize exogenous antigen results in initial appearance of the site T cell determinant occurring ahead of the appearance of newly synthesized HA (Fig. 2 b).

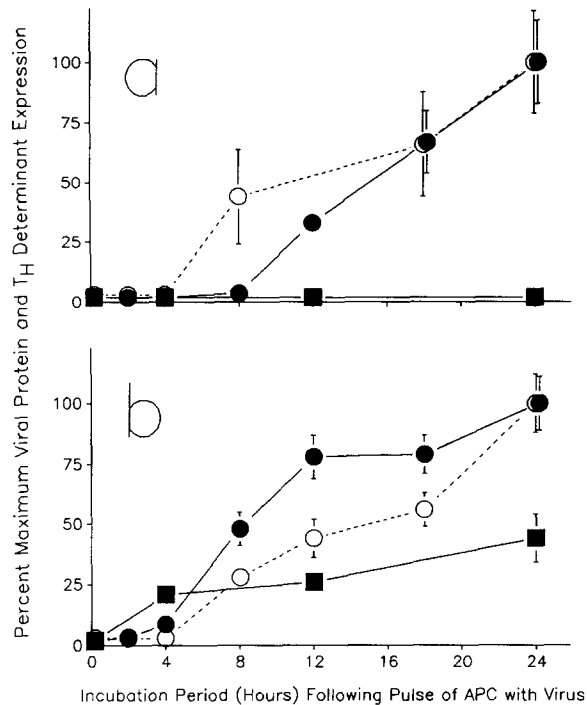


FIGURE 2. Kinetics of expression of de novo synthesized viral proteins and viral Th determinants. APC were pulsed with infectious or UV-inactivated virus and harvested at indicated time points after incubation at 37°C. Aliquots were either paraformaldehyde fixed and placed into T cell stimulation assays, or lysed by sonication and tested for viral protein expression in ELISA. Data are expressed as the percent of maximum HA or NA protein expression or T cell stimulation obtained over the time course of the experiment. (a) Expression of NA protein (O) and recognition by NA-specific T cell NA-2.10 of APC pulsed with infectious (●) or UV-treated virus (■). (b) Expression of HA protein (O) and recognition by HA/site 1-specific 3.2B11 of APC pulsed with infectious (●) or UV-treated virus (■).

NA T Cell Determinant Expression by Infected APC Does not Correlate with Production of a Greater Quantity of NA in Infected Cells than Is Available on Nonreplicative Virions. The amount of NA present on virions and within infected A20 cells was quantitated. Preparation of ultrasonicated and purified virus was mixed with a lysate of uninfected A20 cells and compared by ELISA with lysates of A20 cells 24 h after infection with influenza virus. Table I shows that addition of 10^3 HAU of UV-treated virions results in an 80-fold higher concentration of NA than arises in cells by infection, yet is unable to elicit recognition by the NA-specific clone.

The T Cell Determinant Is Present in NA Isolated from Egg-grown Influenza Virions; Recognition of Isolated Molecules May Bypass Cellular Processing Requirements. To test whether a neo-antigen related to NA is recognized, NA was isolated from purified egg-grown PR8 virus and tested for its ability to be recognized by the NA-specific T cell clone. A preparation of detergent-solubilized HA/NA glycoproteins free of infectious virus was recognized by the NA-specific T cell clone in concentrations ranging from ~ 10 – 100 -nM equivalents of the NA protein monomer (Table II). Although these are much higher concentrations of NA than observed in stimulatory infected cell cultures, concentrations of NA on UV-treated virions within this range were non-stimulatory (compare with Table I).

The ability of extracted NA glycoprotein to be recognized by NA-specific T cells that do not respond to nonreplicative virus was studied further. Table III shows that NA as part of the glycoprotein preparation is presentable by paraformaldehyde-prefixed A20 cells, with or without reduction and alkylation of the preparation. Although

TABLE I
*UV-inactivated Virions Containing a Higher NA Content than
Infected APC Fail to Stimulate NA-specific T Cells*

Antigen source	Antigen concentration* in culture of:		Stimulation (OD ₅₇₀) of T cells recognizing: [†]	
	HA	NA	HA	NA
	<i>nM</i>			
UV virus	136	25	0.263	0.010
Infected cells	15	0.3	0.250	0.225

* Viral protein content was found to be 18.9 ng/HAU for HA and 270 pg/HAU for NA by comparing ELISA values of purified virus with those obtained with purified preparations of each glycoprotein at known concentration. Infected A20 cells as used in the assay contained a virus equivalent of 280 HAU per 10⁷ cells. Molecular masses of HA and NA protein monomers used in calculations were 70 and 50 kD, respectively (39). Antigen concentrations reflect presence in cultures of 5 × 10³ HAU/ml UV virus or 2 × 10⁶/ml infected APC.

[†] T cell assays were performed with UV-treated virus or infected APC continuously present in stimulation cultures. OD₅₇₀ nm values reflect IL-2-dependent growth of CTLL indicator cells, with assay background in absence of antigen giving OC₅₇₀ of 0.01–0.02.

TABLE II
*The Determinant Recognized by the NA-specific T Cell Clone Is
Present on Glycoprotein Purified from Egg-grown Influenza Virus*

Concentration of NA monomer in culture*	Stimulation (OD ₅₇₀) of NA-specific T hybrid
<i>nM</i>	
132	0.317
66	0.345
33	0.303
16	0.191
8	0.010
4	0.005

* Concentration of NA was determined by comparing ELISA values using NA-specific mAbs obtained with the glycoprotein preparation to that of purified NA of known protein concentration.

TABLE III
*Recognition of Isolated Influenza Glycoprotein Presented by
Untreated or Prefixed A20 Cells*

T hybridoma	T cell stimulation (OD ₅₇₀) in presence of:*			
	Untreated APC		Pre-fixed APC	
	Untreated HA/NA	Reduced and alkylated HA/NA	Untreated HA/NA	Reduced and alkylated HA/NA
NA-2.10	0.446	0.148	0.145	0.099
3.2B11	0.298	0.424	0.034	0.224

* HA/NA rosettes were purified from virus and an aliquot reduced and alkylated as described in Materials and Methods. 1:100 final dilutions (34 µg/ml) of these preparations were cocultured with APC, untreated or pre-fixed, and the HA/site 1-specific 3.2B11 or the NA-specific NA-2.10 in a standard T cell stimulation assay. Responses threefold above assay background are underscored.

the level of T cell stimulation is reduced on prefixed APC, this is seen also with an HA-specific T cell, which differs from the NA-recognizing clone in requiring reduction and alkylation for sensitization of prefixed APC (Table III). Intact purified virus added to prefixed APC was not recognized by either HA- or NA-specific T cell clones (not shown). These results suggest that the glycoprotein extraction procedure may have the effect of "pre-processing" the region of NA that contains the Th determinant, thereby allowing recognition of isolated molecules in a way not possible for intact virions.

Discussion

Class II MHC-restricted T cells specific for influenza virus structural proteins were identified that respond much better to infectious than to noninfectious virus. Expression of a T cell determinant of the NA glycoprotein most strongly correlated with viral infection. Several observations indicate that the protein of exogenous virus does not significantly contribute to formation of that determinant. Firstly, NA-specific T cell recognition was observed to trail the synthesis of viral NA within infected APC. Secondly, quantitative studies showed that higher NA concentrations in cultures could be achieved by addition of UV-treated virus than occurs by synthesis within infected cells, yet without resulting in T cell recognition. Finally, NA extracted by detergent treatment of egg-grown virions was recognized by T cells; however, this preparation was able to sensitize pre-fixed APC from without, indicating that the treated protein no longer required antigen handling by the APC. This suggests that although the NA determinant is cryptically present on exogenous virions, the normal antigen-handling pathway is ineffective in leading to its presentation.

Further, it seems unlikely that the concentration of antigen within infected cells underlies the effectiveness of infectious virus. The major evidence for this is the differential effect of infectivity observed for two T cell sites of the HA molecule. Expression of site 3 was enhanced ~10-fold on infected APC, while at the same time T cell recognition of HA site 1 was only slightly better than with an equivalent amount of UV-treated virus. Both determinants, located on the same HA1 polypeptide, should be produced in equimolar amounts within infected cells, implicating properties other than intracellular concentration in determining the relative contribution of endogenously synthesized antigen to T cell recognition.

Available evidence suggests that the T cell sites that show greatest enhancement as a result of endogenous viral protein synthesis are the more labile determinants. Considering HA sites 1 and 3, although both are linear determinants that require APC function inhibitable by lysosomotropic agents or aldehyde prefixation for their generation from native virus (11), previous studies have shown that processing requirements and expression characteristics of the two I-E^d-restricted determinants greatly differ. Firstly, presence of the diffusible protease inhibitor leupeptin decreases presentation of site 1 while enhancing site 3 expression several fold (34). Secondly, transient exposure of virus to pH 5, similar to the acidity encountered in endosomes, efficiently sensitizes pre-fixed APC for recognition of site 3 but is not sufficient for recognition of site 1 (35). Finally, studies of the kinetics of determinant expression show that site 3 is expressed at maximal levels ~8 h post-pulse with nonreplicative virus and declines greatly by 48 h, while site 1 expression increases continuously over that entire time period (34). From these observations, site 3 appears to have

fewer enzymatic requirements for its presentation than site 1, while also being sensitive to destruction by leupeptin-inhibitable cellular enzymes and more readily turned over by active APC. Although less information is available for the NA site, preliminary observations suggest that it may be relatively labile. Detergent extraction of virion NA, while maintaining NA enzymatic activity, results in a "pre-processing" of the molecule for T cell recognition. However, this preparation lost its ability to sensitize pre-fixed APC for T cell recognition when briefly exposed to pH 5 under conditions of calcium chelation (data not shown), which destroys sialidase activity of subtype N1 NA (8). One speculation is that a presentation pathway linked to synthesis may bypass the destructive effects of the endosome-lysosome system and thereby enhance the levels of labile determinants. Alternatively, determinants that have short half-lives on APC surfaces may benefit by being continuously replaced from newly synthesized material in infected APC, without invoking a unique pathway. It seems likely that all T cell determinants are presented by infected APC, but that the greatest contrast is observed with those that survive less well when introduced on a single pulse of antigen.

To date no direct evidence is available that nonoverlapping pathways are used to present endogenous vs. exogenous antigen in association with MHC class II, or that the endogenous route corresponds with that proposed for generation of class I MHC-restricted determinants. Inconclusive results were obtained with chloroquine treatment, often used to disable endosomal pathways (6, 10, 16, 36-38), since its addition at intervals after infection greatly decreased viral protein expression in infected cells (data not shown) upon which recognition was shown to depend (Fig. 2). Mapping of the routes utilized may depend upon availability of inhibitors with more narrow specificities. It is also possible that some apparent distinctions in the generation of class I and class II MHC-restricted determinants may involve differences in kinetics of their appearance. For example, the observation by Morrison et al. (16) of HA-specific class I- but not class II-restricted lysis of recombinant vaccinia-infected targets 6-7 h post-infection could reflect a delayed appearance of class II target structures. It may be relevant that our NA determinant was nearly undetectable until after 8 h post-infection with influenza virus.

The varying dependencies of class II-restricted Th determinants upon an endogenous source of antigen may be relevant to vaccination. Immunization with replication-inactive virus may fail to elicit the same range of Th as results from the usual encounter with infectious virus. This could conceivably lead to a compromised response of the Th compartment when challenged by natural infection.

Summary

The contribution of viral infectivity to the expression of MHC class II-restricted T cell determinants was studied. A murine I-E^d-restricted T cell hybridoma recognizing the neuraminidase (NA) glycoprotein of influenza PR8 virus was stimulated strongly by infectious virus but failed to recognize antigen introduced on noninfectious virions. Recognition correlated with the de novo synthesis of viral NA within infected APC. The effectiveness of infectious virus did not depend strictly upon the amount of NA present in cultures, since high NA concentrations could be achieved by addition of nonreplicative virus without being stimulatory for NA-specific T cells. Recognition of a determinant generated only when synthesized in murine host cells

was ruled out, since, in high concentration, NA isolated from purified egg-grown virions, even if reduced and alkylated, was recognized by the T hybridoma clone. Isolated NA was recognized when added to pre-fixed APC, suggesting that this form of antigen was able to bypass the usual processing pathway of exogenous proteins. Data suggest that endogenously synthesized antigen may contribute most significantly to presentation of labile T cell determinants. In addition to NA, recognition of an I-E^d-restricted determinant of the influenza hemagglutinin (HA) molecule, shown previously to have a relatively short half-life on APC surfaces, was enhanced greatly by infectious virus. In contrast, T cell recognition of a more stably expressed I-E^d-restricted site of the same HA polypeptide was only marginally improved on infected APC.

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