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Immunogenic Peptides of Influenza Virus Subtype N1 Neuraminidase Identify a T-Cell Determinant Used in Class II Major Histocompatibility Complex-Restricted Responses to Infectious Virus

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Six nonoverlapping peptides of the neuraminidase (NA) glycoprotein of influenza virus A/Puerto Rico/8/34 (H1N1) (PR8 virus) were found to be immunogenic for proliferating T cells when injected into BALB/c mice in Freund adjuvant. T cells elicited by peptide immunization could recognize PR8 virus in vitro. However, only one of these peptides, corresponding to residues 79 to 93 of NA (NA 79–93), was able to restimulate T cells of mice immunized with infectious virus. T cells that recognized this peptide were uniformly I-E^d restricted, yet infectious influenza virus was required for responses. NA 79–93-specific T-hybridoma clones raised by immunization either with whole virus or with the synthetic peptide alone each responded to replicative virus and not to UV-inactivated virions. These data suggest that the NA 79–93 T-cell determinant which is commonly presented during an encounter with influenza virus in vivo is processed preferentially from NA synthesized within antigen-presenting cells.

The neuraminidase (NA) molecule of type A influenza viruses is a glycoprotein of the virion envelope which, along with the hemagglutinin (HA), is a major target of antiviral immune responses. Like HA, the NA molecule is subject to antigenic shift and drift variation (1, 2, 23), with human influenza viruses carrying either subtype N1 or N2 molecules (11). The three-dimensional structure of the subtype N2 NA has been described previously (10, 35), including details of its interaction with NA-specific monoclonal antibodies (9). Much less information is available on the recognition of NA by T cells. Class I major histocompatibility complex (MHC)-restricted cytolytic T lymphocytes have recently been described for murine responses to N1 (36) and N2 (31) strains. Class II-restricted T cells to NA have been described in humans (26) and mice (21); however, the location of T-cell antigenic determinants of NA have not previously been reported.

We have been studying the recognition of the subtype N1 NA molecule by murine class II MHC-restricted T cells. NA-specific Ia-restricted T-hybridoma clones have been reported which differ from most class II-restricted T cells in requiring viral biosynthesis within antigen-presenting cells for antigen recognition, failing to respond to nonreplicative virus (13). In this study, we used peptide synthesis to identify T-cell antigenic regions of the molecule and to derive T cells to further examine the requirement for viral protein biosynthesis in T-cell recognition of NA.

We report that six nonoverlapping regions of the NA molecule of the subtype H1N1 influenza virus A/Puerto Rico/8/34 (PR8 virus) are immunogenic for class II-restricted T cells when inoculated as peptides into BALB/c mice. One of these peptides appears to represent a major T-cell determinant utilized in the murine response to NA after immunization with intact virus. A requirement for live virus in presentation of that epitope was observed.

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MATERIALS AND METHODS

Viruses. Influenza viruses A/Puerto Rico/8/34 H1N1 (PR8 virus) and B/Lee/40 were grown in the allantoic cavities of embryonated chicken eggs, and their concentration in hemagglutinating units (HAU) was determined by chicken erythrocyte agglutination (14). UV-light inactivation of viral replication ability was achieved by exposure of virus to short-wave UV light (UVGLD58; Ultraviolet Products, San Gabriel, Calif.) at 240 $\mu W/cm^2$ for 15 min. Effectiveness of inactivation was assessed by infecting murine L929 fibroblasts and staining for expression of the nonstructural protein NS-1 of influenza virus with an antibody provided by J. Yewdell (National Institutes of Health, Rockville, Md.).

Synthetic peptides. Peptides were synthesized according to the amino acid sequence of PR8 NA (15) by solid-phase methods. Regions were selected for synthesis by identifying sequences of NA which had low average structural dissimilarity with two previously described I-E^d-restricted determinants of influenza virus PR8 HA (HA1 residues 111 to 119 and 302 to 313) (12, 21). The sequence comparison method of Padlan (30) was used as applied by Sette et al. (32). The entire amino acid sequence of NA was compared residue for residue with each of the standard HA determinants. These were ranked from lowest to highest average structural dissimilarity scores. Regions scoring in the top 5% according to lowest dissimilarity with each of the HA peptides were initially chosen for synthesis. In some cases, several adjacent regions having low scores were synthesized as a single peptide.

Immunization and in vitro T-cell stimulation assays. (i) Virus immunizations. Female BALB/c mice were inoculated intraperitoneally with 500 HAU of PR8 virus per mouse as infectious allantoic fluid or with 5,000 HAU of UV-inactivated purified virus per mouse. In some studies, mice primed with infectious virus were boosted 3 weeks later with recom-

binant vaccinia virus (33) bearing the influenza virus N1 NA gene (NA-Vac). Seven to 14 days later, spleen cells were taken for in vitro assays.

(ii) Peptide immunizations. Female BALB/c mice were injected in hind footpads and intraperitoneally with 10 μg of peptide emulsified in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) per injection site. Ten to 20 days later, the draining lymph nodes were removed and assayed for T-cell proliferation.

(iii) T-cell proliferation assays. Spleen or lymph node cells were cultured at 4×10^6 cells per ml in microtiter wells in Iscove modified Dulbecco medium (IMDM) supplemented with 1% normal mouse serum or 1% nutridoma-SP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and different concentrations of antigens as indicated in Results. For some experiments, a two-stage assay was performed by the method of Chain et al. (8) in which cultures established as above were shifted after 3 days to IMDM containing 5% fetal bovine serum and 2% supernatant of concanavalin A-stimulated rat spleen cells for an additional 2 days. Data were obtained by pulsing cultures with [^{3}H]thymidine (0.5 μCi) for 18 h and liquid scintillation counting to estimate incorporation of radiolabel into DNA.

(iv) T-hybridoma assays. T hybridomas were produced by previously reported methods (19, 21) with cells from immunized mice restimulated in vitro with antigens as noted in Results and fusing with BW5147 cells. All cells were cultured in IMDM containing 5% (vol/vol) fetal bovine serum. The T hybridoma to PR8 HA sequence 111 to 119, I-E^d restricted, has been described previously (21).

Interleukin-2 (IL-2) release by T hybridomas in response to antigen was tested as previously described (12) with A20-1.11 (*H-2^d* B-cell lymphoma [24]) as antigen-presenting cells. A20 cells were used at 5×10^4 cells per well plus antigen, and T hybridomas were cultured at 3×10^4 cells per well in serum-free IMDM at 37°C and 6% CO_2 for 48 h. Culture supernatants were assayed for IL-2 produced by stimulated T cells by using the IL-2-dependent indicator cell line CTLL (18). Viability of CTLL following coincubation with the supernatants for 24 to 36 h was quantitated by a modification (20, 34) of the original method of Mosmann (28), using cleavage of the dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, Mo.). Color development was measured with an EL309 plate reader (Biotek, Winooski, Vt.) at 570 nm (750-nm reference wavelength).

RESULTS

Immunogenic peptides of PR8 NA. Potential T-cell epitopes of influenza virus NA were identified by examining the similarity of segments of the molecule to known T-cell determinants. Since an I-E^d-restricted T-cell response to PR8 NA was known (13), structural similarity of the PR8 linear sequence with each of two previously described I-E^d-restricted T-cell determinants of PR8 HA (residues 111 to 119 and 302 to 313 [12]) was evaluated. The procedure of Sette et al. (32) was followed in essential details (see Materials and Methods), resulting in the selection of several regions of NA showing low average structural dissimilarity to the HA epitopes. Where contiguous regions of NA exhibited low dissimilarity values, a single peptide was synthesized to encompass these areas; no attempt was made to define the minimal effective size of each peptide.

A panel of six peptides (Fig. 1) were synthesized and tested for immunogenicity by inoculation into mice in complete Freund adjuvant. Table 1 shows that each peptide was immunogenic, inducing a T-cell proliferative response in BALB/c mice. T cells induced in this way responded to PR8 virus but not to type B/Lee/40 virus nor to an antigenic region of PR8 HA (residues 126 to 138 of HA1). IL-2-secreting T-cell hybridomas specific for each peptide have been obtained (data not shown), confirming that individual T cells responding to these segments can be isolated.

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                    1.
  1 MNPNQKIITI GSICLVVGLI SLILQIGNII SIWISHSIQT GSQNHTGICN
                                2.                    A.
 51 QNIITYKNST WVKDTTSVIL TGNSSLCPIR GWAIYSKDNS IRIGSKGDVF
101 VIREPFISCS HLECRTFFLT QGALLNDRHS NGTVKDRSPY RALMSCPVGE
                                                      3.
151 APSPYNSRFE SVAWSASACH DGMGWLTIGI SGPDNGAVAV LKYNGIITET
                                    4.
201 IKSWRKKILR TQESECACVN GSCFTIMTDG PSDGLASYKI FKIEKGKVTK
251 SIELNAPNSH YEECSCYPDT GKVMCVCRDN WHGSNRPWVS FDQNLDYQIG
301 YICSGVFGDN PRPKDGTGSC GPVYVDGANG VKGFSYRYGN GVWIGRTKSH
                                          5.
351 SSRHGFEMIW DPNGWTETDS KFSVRQDVVA MTDWSGYSGS FVQHPELTGL
        6.
401 DCIRPCFWVE LIRGRPKEKT IWTSASSISF CGVNSDTVDW SWPDGAELPF
451 TIDK
```

FIG. 1. Location in the linear sequence of influenza virus PR8 NA of synthetic peptides used in this study. Peptides (underlined) predicted to have low average structural dissimilarity with previously known I-E^d-restricted T-cell determinants of influenza virus are numbered 1 to 6, while a poorly matching peptide is labeled A. NA sequence is from Fields et al. (15).

A region of NA covering residues 97 to 108 (peptide A, Fig. 1) was synthesized which, in contrast to the above peptides, matched poorly the I-E^d prototype sequences (i.e., had higher average structural dissimilarity values than the panel shown in Table 1). Peptide A failed to elicit proliferating T cells either to itself or to virus after injection into BALB/c mice in complete Freund adjuvant (data not shown). This lack of activity was not due to toxicity since T-cell proliferation to the purified protein derivative of mycobacteria in the Freund adjuvant was not affected by peptide A in the inoculum or in culture (data not shown). Thus, a control peptide is identified in the NA sequence which lacks demonstrable T-cell antigenic activity in BALB/c mice.

Region 79 to 93 of PR8 NA is a determinant utilized in the class II-restricted T-cell response to virus immunization. To test whether immunization with intact virus could stimulate T cells specific for peptides, BALB/c mice were injected intraperitoneally with PR8 virus, either infectious or UV inactivated, and assayed for proliferation in presence of the various peptides in vitro. Immunization with live virus elicited T cells which recognized NA residues 79 to 93 (NA 79–93) but none of the other immunogenic peptides (Table 2). UV-treated virus failed to stimulate responses to any peptide, although the overall priming to PR8 virus was less effective than with replicative virus. Boosting of live-virus-primed mice with infectious recombinant vaccinia virus able to express the subtype N1 NA molecule (NA-Vac) resulted in an enhanced proliferation to NA 79–93 but did not bring up T-cell responses to the other five peptides. Antibody to I-E^d but not to the I-A^d molecule significantly inhibited T-cell proliferation to peptides 79 to 93 (Table 3), revealing that T-cell responses to this segment are limited entirely to the former restriction element. This analysis identifies the region NA 79–93 as a T-cell determinant of the murine class II MHC-restricted response to PR8 virus.

TABLE 1. Proliferative responses of peptide-primed BALB/c mice to immunizing peptides of NA and to influenza viruses

Antigen (concn)[b]	Incorporation of [^{3}H]thymidine (cpm) by T cells of mice immunized with the following peptides of NA[a]:					
	17–26	79–93	198–212	234–256	388–400	408–419
None	4,723	4,026	6,346	6,990	5,644	5,050
Injected peptide (μg/ml)						
50	47,184	37,029	49,340	28,565	18,566	23,655
5	48,284	36,391	28,898	35,963	9,907	27,450
0.5	16,834	24,573	23,064	40,598	5,586	10,735
0.05	11,893	10,642	13,916	14,598	5,366	10,498
PR8 (HAU/ml)						
5	28,313	27,705	27,789	19,128	21,821	16,660
0.5	21,231	15,375	15,606	20,676	10,899	9,400
B/Lee/40 (HAU/ml)						
5	3,636	3,234	7,776	7,946	6,568	4,038
0.5	3,560	5,235	7,452	4,805	6,470	3,440
Peptide HA 126–138 (μg/ml)						
50	5,940	4,881	8,240	8,446	7,367	5,090
5	5,872	5,414	5,888	9,848	7,670	6,055

[a] Mice were immunized with individual synthetic peptides as shown in Fig. 1 in Freund complete adjuvant, and proliferative responses were measured in a two-stage assay as described in Materials and Methods. Counts per minute at least threefold above background values obtained in absence of added antigen in vitro are underscored.

[b] Each mouse was challenged with the same peptide as used for priming. Allantoic fluid containing infectious PR8 or B/Lee/40 virus was used as a source of live virus in cultures. The peptide HA 126–128 is a similarly prepared antigenic peptide differing in primary sequence from the immunizing peptides used in this study.

T cells specific for NA 79–93 recognize infectious but not UV-inactivated PR8 virus. The role of virus infectivity in T-cell recognition of NA was studied. Results of immunization studies (Table 2) indicated that UV-inactivated virus did not readily prime for T cells recognizing NA peptides. Further, we had previously demonstrated NA-specific T cells that responded in vitro to replicative influenza virus but not to inactivated virions nor to infectious virus in presence of protein synthesis inhibitors (13). We therefore examined (i) whether cloned T cells specific for NA 79–93 exhibited differential recognition of infectious versus inactivated virus and (ii) whether the use of infectious virus in selection of the particular T-cell clone influenced its recognition requirements for live virus. Two NA 79–93-specific T-hybridoma clones were produced by different immunization schemes. Hybrid NA2 BC6.1 was obtained from mice immunized in vivo and boosted in vitro with the synthetic peptide, while hybridoma NA7/11 6.1 was from a fusion of T cells of a mouse immunized with whole PR8 virus. Both T hybridomas were selected by ability to secrete IL-2 in the presence of peptide NA 79–93 and were found to be I-E^d restricted by inhibition with anti-Ia antibodies (data not shown). NA 79–93-specific T hybrids, regardless of whether their selection had included use of infectious virus, responded to antigen-presenting cells treated with infectious but not with nonreplicative virus (Fig. 2). In contrast, a T hybridoma specific for I-E^d and influenza virus HA residues 111 to 119 recognized both UV-treated and infectious forms equally well. These data suggest that the requirement for replicative virus in antigen recognition by these class II MHC-restricted T cells reflects presentation of the antigenic determinant rather than the immunization history of the T cells.

TABLE 2. Immunization of BALB/c mice with infectious influenza virus elicits proliferating T cells which recognize NA 79–93

Antigen	Incorporation of [^{3}H]thymidine (cpm) by T cells from mice immunized with[a]:		
	UV-treated PR8 virus	Infectious PR8 virus	Infectious virus, boosted NA-Vac
None	316	783	2,209
PR8 virus	3,786	14,480	16,564
NA peptide			
17–26	443	1,093	1,965
79–93	288	3,510	13,525
198–212	601	1,322	3,733
234–256	372	730	5,009
388–400	450	812	2,161
408–419	360	656	2,781

[a] Mice were immunized intraperitoneally with 5,000 HAU of UV-inactivated purified virus, 500 HAU of infectious PR8 virus allantoic fluid, or infectious allantoic fluid followed 14 days later by infectious recombinant vaccinia virus (NA-Vac) having the gene for influenza NA. Proliferation assays were performed 7 to 9 days after the last immunization. Counts per minute greater than three times background are underlined.

TABLE 3. I-E^d is the restriction element for proliferative T cells of virus-immunized mice which recognize NA 79–93

In vitro antigen[a]	Antibody to class II[b]	Incorporation of [^{3}H]thymidine (cpm)
None	None	838
NA 79–93	None	13,115
NA 79–93	I-A^d	14,948
NA 79–93	I-E^d	3,910
NA 79–93	I-A^d + I-E^d	3,191

[a] BALB/c mice were immunized with infectious PR8 virus allantoic fluid and 2 weeks later boosted with NA-Vac. Spleen cells were assayed for proliferation in vitro 4 weeks later.

[b] Purified monoclonal antibodies MKD6 (22) to I-A^d and 14-4-4 (29) to I-E^d were included in indicated cultures at 10 μg/ml.

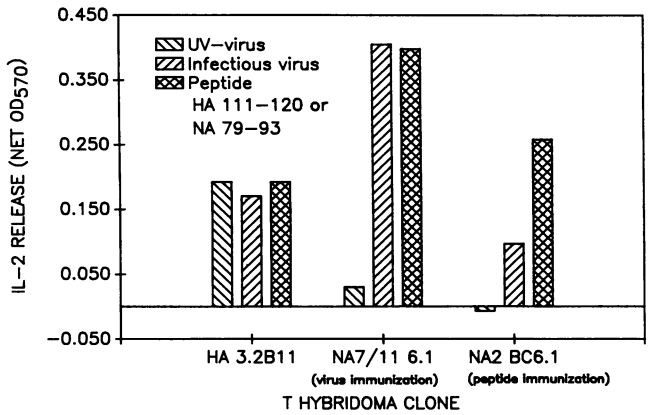

FIG. 2. UV-inactivated virus is not recognized by NA-specific T-hybridoma clones elicited by immunization with virus or peptide. NA 79–93-specific T hybridomas were produced as described in Materials and Methods from mice immunized either with infectious PR8 virus (NA7/11 6.1) or with the synthetic peptide (NA2 BC6.1). Responses (IL-2 release as MTT cleavage by CTLL indicator cells) are compared for recognition of the appropriate synthetic peptide, UV-treated virus, or infectious virus. Ability of UV-treated virus to be recognized is verified by the response of the HA 111–119-specific T-hybridoma clone HA 3.2B11. All T cells are I-E^d restricted.

DISCUSSION

Six regions of influenza virus PR8 NA were identified which can elicit T-cell proliferative responses in BALB/c mice. One of these, the sequence of amino acids 79 to 93 of NA, was found to be a T-cell epitope utilized in the I-E^d-restricted response to influenza virus. By homology to the three-dimensional structures known for other influenza NA molecules (9), the location of the NA 79–93 determinant in the N1 subtype would correspond approximately to a strand of one of the β-sheets (β6) of the subtype N2 head (residues 93 to 108 in that strain). Whether this location is significant in T-cell antigenicity is not known; peptides of this region are immunogenic in vivo and in vitro, but the molecular form of this determinant presented in association with Ia by antigen processing of the NA protein is not resolved.

Of particular interest is that the NA 79–93 T-cell determinant, although linear, class II MHC-restricted, and able to be represented functionally by a synthetic peptide, required viral protein biosynthesis for T-cell recognition. In a previous study, T-cell responses to an as yet unmapped I-E^d-restricted T-cell site of PR8 NA were obtained with infectious virus but not with virions which had been UV treated or with infectious virus added in the presence of protein synthesis inhibitors (13). The quantity of NA produced in infection could not alone account for the antigenic activity of live virus. Results of this study support the hypothesis that the role of viral infection is principally the endogenous production of antigen which somehow favors processing and presentation of the NA 79–93 determinant. T cells elicited only with the synthetic peptide exhibited a requirement for infectivity in recognition of virus similar to that of T cells from virus immunizations; thus, the presentation of the determinant, rather than the immunization histories of the T cells, correlates with the requirement for viral infection in T-cell recognition. The T cells described here should be useful in studying the presentation of endogenously synthesized viral antigens in context of class II MHC, particularly in comparison with presentation of class I MHC-restricted T-cell determinants of influenza virus, which also exhibit a requirement for live virus (17, 27, 37). Knowledge of the amino acid sequences of NA T-cell determinants may be of use in approaching the molecular mechanism for the processing requirements of these sites.

Five regions of NA were found to be immunogenic for virus-reactive T cells when injected as peptides but did not appear to constitute major determinants recognized by virus-immunized mice. This indicates that the selection of NA 79–93-reactive T cells following virus immunization is due neither to inability of the other peptides to associate with MHC nor to lack of appropriate T-cell precursors. Other studies have also shown instances in which peptide immunization may result in recognition of a greater number of sites on a protein than obtained by priming with intact molecules (4, 5, 16). The mechanism by which various antigenic forms elicit T cells specific for different regions of the same protein is not known but may be significant in understanding the basis of immunodominance in immune responses. Peptides provide a high molar concentration of a given T-cell determinant; it could be speculated that T cells of some specificities, either because of low expression of T-cell receptors on precursor cells or because of low receptor affinity, require a large concentration of their target epitope for induction in vivo. Alternatively, antigen processing and presentation of NA may be different in vivo than in vitro. Some insight into these possibilities may come from immunization studies using antigenic forms intermediate between the native molecule and synthetic peptides, e.g., denatured NA glycoprotein or proteolytic fragments. We have shown that detergent-isolated or reduced and alkylated NA can be presented by aldehyde-fixed antigen-presenting cells in vitro, bypassing the requirement for replicative virus (13). We are currently attempting to overcome the limitation in the amount of NA protein that can be purified from influenza virions by using material produced by baculovirus expression vectors for immunization studies.

Estimating the diversity of T-cell recognition of NA requires more extensive analysis. The other glycoprotein of influenza, the HA, has been shown to have multiple class II MHC-restricted T-cell determinants used in murine (3, 6, 7, 21) and human (25) immune responses to various viral subtypes. PR8 virus NA-specific, class II MHC-restricted T-cell clones have been isolated which do not recognize any of the peptides produced so far (data not shown), indicating the presence of additional T-cell sites on the molecule.

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