Class I *H*-2^d-Restricted Cytotoxic T Lymphocytes Recognize the Neuraminidase Glycoprotein of Influenza Virus Subtype N1

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Class I major histocompatibility complex-restricted cytotoxic T lymphocytes (CTL) that recognize the neuraminidase (NA) glycoprotein of subtype N1 influenza A viruses have been demonstrated in BALB/c mice. Responses to NA were obtained only in protocols that use two in vivo inoculations of virus, including a recombinant vaccinia virus containing the NA of subtype N1 influenza virus (NA-VAC) to prime or boost. Restimulation in vitro was also required for CTL recognition of NA and strongly depended on the specific N1 virus used. Influenza viruses A/Puerto Rico/8/34 (H1N1), A/CAM/46 (H1N1), J1 (H3N1), and JAP/BEL (H2N1), but not A/Bellamy (H1N1) or MEM/BEL (H3N1) virus, were able to stimulate NA-specific memory T cells in vitro. Single or double in vivo inoculation of any of the N1 viruses or a single injection of NA-VAC failed to elicit restimulatable NA-specific CTL. Lysis of NA-VAC-infected cells at low effector/target ratios was comparable to that observed toward other influenza virus proteins known to be major targets of CTL in BALB/c mice, indicating that antigenic determinants of the subtype N1 NA molecule can be efficiently presented in the context of major histocompatibility complex class I.

Infection with influenza A viruses elicits class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL) (1, 2, 32, 34). CTL activity has been implicated in reduction of virus shedding (16, 22) and in clearance of virus from infected lungs (20, 21, 31, 38). Definition of target structures recognized by influenza virus-specific CTL has been a major focus of research since the first discovery of CTL (reviewed in references 19 and 40). Early results indicated that the majority of responding CTL tended to be cross-reactive toward any influenza type A virus, while a smaller proportion of the response was specific for the immunizing glycoprotein subtype (e.g., subtype H1N1, H2N2, or H3N2 for human influenza virus strains) (11, 41). By constructing recombinant vaccinia viruses containing isolated influenza virus genes (28), it became possible to determine CTL recognition of target cells expressing individual viral proteins. This approach has confirmed that cross-reactive CTL generally recognized the less variable internal antigens nucleoprotein (NP) (33, 39), matrix protein (M1) (14, 15), polymerases (PB1 and PB2) (3, 4, 7, 26), and nonstructural protein (NS1) (7), while strain-specific CTL tended to be directed toward the surface glycoprotein hemagglutinin (HA) (5, 6, 8, 37). The recognition of the other surface glycoprotein, the neuraminidase (NA), by cross-reactive or strain-specific CTL was speculated upon (18, 29) but only recently shown to occur (27). A CTL response to the subtype N2 molecule in *H*-2^k haplotype mice was detected only after in vivo priming of mice with recombinant vaccinia virus expressing N2 neuraminidase (N2-VAC). The same priming procedure failed to induce N2-specific CTL in *H*-2^d haplotype mice. Subtype N1 NA-specific CTL had not been detected in BALB/c mice in previous studies that used in vivo priming and secondary in vitro boosting with influenza virus (36). It is known that in some haplotypes CTL responses to a given viral protein may be absent due to inability of a particular MHC to present the molecule (7, 25, 30). In mice, for example, PB1 protein is recognized by CTL raised in *H*-2^k but not in *H*-2^d mice, while PB2 presents the

opposite pattern of recognition (7). In addition to the MHC background, it has also been shown that lack of CTL responsiveness to influenza virus proteins could be influenced by the immunization procedures (24).

We were interested in whether CTL responses to subtype N1 NA could be obtained by priming protocols, utilizing a recombinant vaccinia virus containing the gene for subtype N1 influenza virus (NA-VAC). In this paper, we report that recognition of NA by CTL of BALB/c (*H*-2^d) mice can be achieved by in vivo priming or boosting with NA-VAC followed by in vitro restimulation with the appropriate strain of N1 influenza virus.

MATERIALS AND METHODS

Mice. Female BALB/c mice (*H*-2^d), aged 5 to 8 weeks, were purchased from Jackson Laboratory, Bar Harbor, Maine.

Viruses. Influenza viruses A/Puerto Rico/8/34 (PR8; H1N1), A/Cam/46 (A/CAM; H1N1), and A/Bellamy (Bel; H1N1) and reassortant viruses J1 (H3N1), JAP/BEL (H2N1), MEM/BEL (H3N1), and B/Lee/40 were grown in the allantoic cavities of 10-day-old embryonated chicken eggs and stored as infectious stock at -70°C. The recombinant vaccinia viruses that express the influenza virus gene product used in this study have been described previously (28). The indicated genes derived from PR8 were present in the following recombinant vaccinia virus vectors: HA-VAC, NP-VAC, NS1-VAC, M1-VAC, PB1-VAC, PB2-VAC, and PA (acidic polymerase)-VAC. NA-VAC expressed the NA of A/CAM/46, and H2-VAC expressed the HA of A/JAPAN/305/57 (H2N2). Vaccinia virus recombinants were grown in BSC cells, harvested, and stored frozen at -70°C as crude virus stocks in balanced salt solution containing 0.1% bovine serum albumin (13).

Medium. Iscove's modified Dulbecco modified Eagle medium (IMDM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal bovine serum (Hyclone, Logan, Utah) was used for all in vitro cultures.

Cells. P815, a DBA/2 mastocytoma cell line (*H*-2^d), and L-929, a connective tissue cell line derived from a C3H

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(*H-2^k*) mouse, were maintained in IMDM with 5% fetal bovine serum.

Immunizations. Mice were primed with 300 to 500 hemagglutinating units of PR8 or J1 virus intraperitoneally. Two weeks later, mice were boosted with 2×10^7 PFU of NA-VAC intraperitoneally. To prime with NA-VAC, mice were injected with 2×10^7 PFU intravenously.

Secondary in vitro culture. Splenocytes from mice immunized at least 2 weeks previously with virus were restimulated in vitro. Stimulator cells originating from autologous splenocyte populations were infected with virus for 1 h and then mixed with responder populations in a 2:1 ratio and incubated in a CO₂ incubator for 6 days.

Cytotoxicity assay. Cytotoxicity was assayed by using a ⁵¹Cr release assay. Targets were prepared by infecting 2×10^6 P815 cells with 1,000 to 1,600 hemagglutinating units of influenza viruses or 2×10^8 PFU of recombinant vaccinia viruses (VAC). After 1 h of incubation at 37°C, targets were washed and incubated overnight at 32°C in 7 ml of IMDM. For ⁵¹Cr labeling, 250 µCi of Na⁵¹CrO₄ was added to cells in IMDM, followed by incubation for 1 h at 37°C. After two washes, 10^4 radiolabeled targets were added to each well of a 96-well, round-bottom microdilution plate. Effector T cells from stimulation cultures were added to give effector/target ratios of 10:1, 5:1, 2.5:1, and 1:1 in a 200-µl final volume. After 4 h of incubation in a 37°C CO₂ incubator, 100 µl of supernatant was harvested and counted in a Rackgamma counter (LKB Instruments Inc., Rockville, Md.). The percent specific ⁵¹Cr release was calculated as [(experimental release - spontaneous release)/(total release - spontaneous release)] × 100.

To verify that the NA molecule was expressed on the surface of infected cells, flow cytometric analysis (fluorescence-activated cell sorter) of P815 cells infected with PR8, J1, or NA-VAC virus was performed. Infected cells were incubated with biotinylated monoclonal anti-HA or anti-NA antibody (provided by W. Gerhard, Wistar Institute) for 20 min at 4°C, followed by incubation with fluorescein isothiocyanate-avidin for 5 min at 4°C. Results showed that 32% of PR8-infected cells bound anti-HA antibody while 8% bound anti-NA antibody. J1- and NA-VAC-infected cells were negative for subtype H1 HA expression, while 24 and 17% of cells, respectively, showed specific binding of anti-NA antibody.

RESULTS

The NA molecule is a target for class I MHC-restricted CTL. We have established an immunization protocol to induce murine class I MHC-restricted CTL specific for the NA molecule of influenza N1 subtype viruses. In the optimal protocol, spleen cells taken from BALB/c mice primed with influenza virus PR8, followed by boosting in vivo with NA-VAC, were restimulated in vitro with J1 (H3N1) influenza virus for 6 days. Cultures were examined in a ⁵¹Cr release assay for the ability to recognize infected P815 targets, which express class I but not class II MHC antigens. Figure 1 shows that effector cells expressed cytolytic activity against NA-VAC-infected targets but not against target cells infected with the irrelevant H2-VAC recombinant, ruling out recognition of vaccinia virus antigens in this response. Cytotoxic cells induced in this manner also lysed P815 cells infected with PR8 or J1 virus (Fig. 1) but not those infected with type B/Lee influenza virus (data not shown), demonstrating that they possess the type specificity characteristic of influenza virus-specific CTL. No significant lysis

FIG. 1. Recognition of the NA molecule by PR8 (primed)-, NA-VAC (boosted in vivo)-, and J1 (in vitro)-induced effectors. ⁵¹Cr release assay was performed on P815 cells infected with the indicated viruses. Spontaneous release from all targets was 5 to 15%. Symbols: □, uninfected; ●, PR8; ○, J1; △, NA-VAC; ■, H2-VAC; ▲, J1-UV.

of target cells treated with UV-inactivated J1 virus was observed, in agreement with the generally accepted finding that de novo synthesis of viral proteins is required for class I MHC-restricted target formation (9). Class I MHC restriction of NA-specific effector cells was further evidenced by lack of lytic activity toward NA-VAC-infected L-929 cells of *H-2^k* origin (data not shown). The T-cell phenotype of CTL has been confirmed by the ability of anti-Thy-1.2 antibody and complement to abolish lytic activity of the effector population toward influenza virus-infected target cells (data not shown).

Among the CTL induced in the above immunization protocol are T cells specific for various proteins of influenza virus. Table 1 shows that lysis of NA-VAC targets compares well with that observed against NP, NS1, and PB2, which have been known previously to be excellent CTL targets. In the protocol that uses J1 stimulation, CTL to subtype H1 HA are not induced, although PR8 HA is known to be a target of *H-2^d*-restricted CTL (6).

Induction of NA-specific CTL is dependent on in vivo immunization with NA-VAC and on the strain of N1 influenza virus used for in vitro stimulation. (i) In vivo stimulation. It was notable that not all immunization protocols led to CTL recognition of NA-VAC. Only splenocytes from mice primed with PR8 and boosted with NA-VAC, or primed with NA-VAC followed by boosting with N1 influenza virus, demonstrated significant CTL activity against NA (Table 2).

TABLE 1. Recognition of influenza virus proteins by effector T cells from sequential immunizations with PR8, NA-VAC, and J1 (in vitro)^a

Targets	% Specific ⁵¹ Cr release from infected targets at given effector/target ratio	
	10:1	2:1
J1	64	27
H1-VAC	0	0
NP-VAC	66	44
PB2-VAC	39	14
PB1-VAC	14	0
PA-VAC	21	4
M1-VAC	7	2
NS1-VAC	78	28
NA-VAC	64	40
VAC	4	0

^a Lysis of uninfected targets was approximately equal to lysis obtained with VAC-infected cells.

TABLE 2. Recognition of the NA molecule by CTL induced by different immunization procedures^a

Expt	Stimulation			% Specific ⁵¹ Cr release from targets infected with:	
	In vivo		In vitro		
	1	2		J1	NA-VAC
1	PR8		J1	62	5
	PR8	NA-VAC	J1	67	69
2	PR8	J1	PR8	56	0
	J1	PR8	J1	56	0
	NA-VAC	PR8	J1	43	29

^a First and second stimulations took place in vivo; the third stimulation was performed in vitro with the indicated viruses. Results were obtained at an effector/target ratio of 10:1. Data represent percentage of specific killing after subtraction of values indicating lysis of uninfected targets.

Priming and boosting with only PR8 and J1 influenza viruses was not sufficient to demonstrate CTL specific for NA-VAC-infected targets. Single in vivo priming of mice with N1 influenza viruses or with the NA-VAC recombinant (data not shown), followed by in vitro restimulation, repeatedly failed to induce NA-specific CTL. These results show that immunization with NA-VAC was a critical element in achieving a response against the NA molecule.

(ii) **In vitro stimulation.** The strain of subtype N1 influenza virus used for in vitro restimulation was found to be another important factor in inducing NA-specific CTL. Results presented in Table 3 demonstrate that PR8, A/CAM, and J1 viruses strongly stimulate NA-specific responses. In contrast, neither BEL nor MEM/BEL virus (carrying the NA of BEL) was able to stimulate NA-specific CTL. BEL virus was a poor stimulator of virus-specific responses in general (11, 41), while MEM/BEL primed for secondary in vitro CTL that recognize J1 virus. Nevertheless, JAP/BEL (H2N1), also expressing the NA of BEL, showed strong ability to stimulate NA-specific memory CTL. These data show that individual N1 viruses can have greatly differing abilities to stimulate NA-specific CTL. Results with reassortants bearing the NA gene derived from BEL suggest that immunogenicity of NA for CTL may depend on the viral context in which the molecule is delivered.

TABLE 3. Differential effect of N1 subtype viruses on stimulation of NA-specific memory CTL^a

Stimulation in vitro with:	% Specific ⁵¹ Cr release from targets infected with:			
	NA-VAC		J1	
	10:1	2.5:1	10:1	2.5:1
Expt 1				
PR8	32	27	66	34
A/CAM/46	40	30	48	38
J1	77	68	75	65
Expt 2				
JAP/BEL	48	47	64	58
MEM/BEL	0	6	31	23
BEL	0	0	6	3
J1	53	41	64	43

^a Cytolysis of uninfected targets has been subtracted from the values. Results were obtained at the indicated effector/target ratios.

DISCUSSION

Class I MHC-restricted CTL specific for the influenza virus subtype N1 glycoprotein were elicited by sequential stimulation of BALB/c mice with different combinations of subtype N1-expressing viruses. Induction of NA recognition by CTL was obtained only when an NA-VAC recombinant was used to prime or boost mice in vivo. Our previous experience (36) and data presented in Table 2 indicate that neither the combination of PR8 and J1 viruses nor NA-VAC by itself was able to stimulate detectable levels of NA-specific CTL. In vivo boosting had been shown to increase overall activity of influenza virus-specific CTL (11). The requirement for in vivo boosting in our study may indicate the presence of relatively few NA-specific precursor CTL, requiring a repeat stimulation to yield appreciable numbers of memory cells. The importance of NA-VAC in such a priming scheme is not well understood. Expression of NA is linked in this system to the infectivity of the VAC toward murine host cells. One possibility is that a greater overall quantity of NA may be available following NA-VAC infection than is achieved with influenza virus, thereby enhancing availability of the molecule to CTL precursors. Also, as a consequence of being the only influenza virus protein expressed on cells infected with the VAC, NA may be more readily available for T-cell recognition than when other influenza virus proteins are present in the same cell. NA protein is known to be expressed on influenza virions and virus-infected cells at only about 2 to 20% of the level of HA (12, 35). Alternatively, the synthesis of NA by the VAC may in some way favor the processing and presentation of epitopes in the context of class I MHC.

Results presented in Table 3 demonstrate that not all N1 influenza viruses are able to stimulate NA-specific memory CTL in vitro. BEL virus and the reassortant MEM/BEL (H3N1) carrying the BEL NA did not elicit NA-specific CTL. In several experiments that utilized BEL virus in secondary in vitro stimulation, we have observed either a general lack of CTL responses or very low specific killing with a high level of nonspecific lysis on uninfected targets (data not shown). BEL virus had been shown in other studies to be a poor stimulator of murine CTL (11, 41). The virus is able to adequately biosynthesize proteins in murine cells, since about 71% of P815 mouse mastocytoma cells infected with BEL virus stained positive with a monoclonal antibody to NA, comparable to other viruses which were better CTL stimulators (not illustrated). The JAP/BEL (H2N1) reassortant, also having the NA of BEL, stimulated strong CTL responses against NA, comparable to responses induced by A/CAM/46, PR8, and J1 viruses. It is possible that the NA of JAP/BEL underwent changes that made it more accessible for presentation to T cells. Alternatively, the viral gene context in which NA is delivered may greatly alter its immunogenicity. Experiments that use genetic vectors to study the expression of NA CTL determinants as a function of the presence or absence of different individual influenza virus proteins would be useful in understanding the potency of different viruses in CTL induction.

Immune responses to influenza virus NA have been shown to exhibit a number of unusual features. Kilbourne and collaborators attributed the inability to induce optimal antibody responses to NA to a form of antigenic competition in which HA was dominant over NA when introduced on the same virus particle (17). Antibody level could be increased by cross-boosting with the same NA in the context of a different HA subtype. It is not clear whether a similar

phenomenon is operative in our case, since cross-boosting with influenza viruses having different HA molecules is routinely required to obtain *in vivo* boosting of CTL (11), most likely by permitting the viral infectivity needed in formation of class I MHC-associated target structures. Also of interest is the recent characterization of class II MHC-restricted T cells specific for NA of influenza virus PR8 which require viral protein biosynthesis for antigen recognition (12), in contrast to previously described Ia-restricted T cells which preferentially recognize influenza virus antigens introduced exogenously on inert virus particles (23). Together with results reported here that class I-restricted recognition of NA also requires replicative virus, the possibility arises that both helper and CTL to NA have similar requirements for induction in relation to virus infectivity and circulating neutralizing antibody. However, no information is available on the possible role of NA-specific T helper cells in development of NA-specific CTL *in vivo*.

Demonstration of CTL responses to NA is of importance in understanding immune recognition of influenza virus. Characterization of the role of viral glycoproteins in eliciting cross-reactive and strain-specific cellular immune responses to influenza virus should include both HA and NA. NA represents a target antigen more highly conserved in human influenza virus strains than the HA, but less conserved than internal proteins of the virus. Information is needed on the epitopes of NA recognized by CTL and whether CTL responses to this antigen can play a role in immunity or recovery from influenza virus infection.

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