Anti-Influenza Virus Cytotoxic T Lymphocytes Recognize the Three Viral Polymerases and a Nonstructural Protein: Responsiveness to Individual Viral Antigens Is Major Histocompatibility Complex Controlled

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It has recently been shown that antiviral major histocompatibility complex class I-restricted cytotoxic T lymphocytes can recognize proteins that serve as internal viral structural components (influenza A virus nucleoprotein, vesicular stomatitis virus nucleocapsid protein). To further examine the role of internal viral proteins in cytotoxic T-lymphocyte recognition, we constructed recombinant vaccinia viruses containing individual influenza A virus genes encoding three viral polymerases (PB1, PB2, PA) and a protein not incorporated into virions (NS1). We found that cells infected with each of these recombinant vaccinia viruses could be lysed by anti-influenza cytotoxic T lymphocytes. Cytotoxic T-lymphocyte responsiveness to the individual viral antigens varied greatly between mouse strains. By using congenic mouse strains, responsiveness to PB1 and PB2 was found to cosegregate with major histocompatibility complex haplotype. These findings provide further evidence that internal antigens play a critical role in cytotoxic T-lymphocyte recognition of virus-infected cells. Additionally, they suggest that the cytotoxic T-lymphocyte response to viral antigens may often be restricted to only a fraction of the major histocompatibility complex class I repertoire.

Class I major histocompatibility complex (MHC) restricted cytotoxic T lymphocytes (CTLs) arise during infections with many viruses and are believed to play an important role in eradicating established infections. A prominent feature of the CTL response is its cross-reactivity for different virus serotypes. In the case of influenza virus, it was found in early studies that a large portion of both murine (10, 39, 43) and human (4, 21) anti-influenza virus CTLs recognize cells infected with influenza A viruses derived from any of the three human subtypes. (Human type A influenza viruses are grouped into three subtypes, designated H1N1, H2N2, and H3N2. H1, H2, and H3 and N1 and N2 each designate non-cross-reactive serotypes of the hemagglutinin [HA] and neuraminidase molecules, respectively.) These CTLs were termed cross-reactive, while those which only recognized cells infected with the immunizing subtype or closely related strains were termed strain-specific. While it was clear from these studies that at least a portion of strain-specific CTLs recognized the HA, it proved extremely difficult to identify the viral proteins recognized by cross-reactive CTLs.

Recently, it has been possible to directly examine CTL recognition of individual viral proteins by using target cells expressing cloned viral genes. Using this approach it was found that the HA molecule is primarily recognized by strain-specific CTLs and is not a major target structure for cross-reactive CTLs (2, 6, 34) and that a major population of cross-reactive CTLs recognize the nucleoprotein (NP), an internal virion structural protein that is located predominantly in the nucleus and cytoplasm of infected cells (34, 41).

Based on results of unlabeled target inhibition studies (41), it appeared that cross-reactive CTLs might recognize other viral antigens in addition to NP.

To examine this possibility, we produced recombinant vaccinia (VAC) viruses containing cloned influenza virus genes encoding the three influenza virus polymerases (PA, PB1, PB2). The polymerases are synthesized in relatively low amounts during the infectious cycle and are present in small quantities in virions. As their names indicate, they are known to be involved in replication and transcription of viral RNA (16). One of these proteins (PB2) was implicated as a CTL target structure in a previous study (1). We also produced a recombinant VAC virus containing the gene encoding an abundantly produced protein of unknown function (18) which is believed to be completely excluded from virions (nonstructural 1 [NS1]). The possibility that CTLs specific for nonstructural viral proteins arise in the immune response to a virus infection has not been examined previously.

We found that each of these four proteins can be recognized by anti-influenza virus CTLs. Recognition of the individual proteins varied greatly between mouse strains, however. Using congenic mouse strains, we show that recognition of individual components cosegregates with MHC haplotype.

MATERIALS AND METHODS

Mice. Male BALB/c (*H-2^d*), C57BL/6 (*H-2^b*), CBA/J (*H-2^k*), B10.D2 (*H-2^d*), and B10.Br (*H-2^k*) mice (age, 8 to 12 weeks) were purchased from Jackson Laboratory, Bar Harbor, Maine. BALB/K mice were kindly provided by K. Blank of the University of Pennsylvania, Philadelphia, Pa.

Viruses. Influenza virus PR8 (A/Puerto Rico/8/34[H1N1]), JAP (A/Japan/305/57[H2N2]), NT60 (A/Northern Ter-

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TABLE 1. Recognition of P815 cells expressing isolated influenza virus antigens by BALB/c cross-reactive anti-influenza virus CTLs

P815 cells	Percent specific ⁵¹ Cr release from:					
	PR8/JAP ^a			PR8/HK ^a		
	7:1	2:1	1:1	7:1	2:1	1:1
PR8	89	85	65	84	76	56
PB1-VAC	31	19	9	16	8	3
PB2-VAC	78	48	20	83	61	29
PA-VAC	66	37	14	26	13	4
NS1-VAC	88	83	59	88	76	41
NP-VAC	96	97	90	96	97	80
VAC	17	8	5	10	6	4

^a The first strain designation is primary in vivo stimulation; the second strain designation is secondary in vitro stimulation. Cytotoxic assays were performed with BALB/c (*H-2^d*) splenocytes at the indicated effector:target ratios.

ritory/60/68(H3N2)), and HK (A/Hong Kong/1/68(H3N2)) were grown in the allantoic cavity of 10-day-old embryonated chicken eggs and stored as infectious stocks at -70°C . The construction of VAC recombinant viruses that express foreign virus gene products has been described previously (19, 30). The recombinant VAC viruses used in this study (and the foreign gene products that they expressed) are as follow: PB1-VAC (influenza PR8 basic polymerase 1 [PB1]); PB2-VAC (influenza PR8 basic polymerase 2 [PB2]); PA-VAC (influenza PR8 acidic polymerase [PA]); NS1-VAC (influenza PR8 NS1); NP-VAC (influenza PR8 NP).

All influenza virus genes were kindly provided by P. Palese, Mt. Sinai School of Medicine, New York, N.Y. VAC viruses were grown in BSC cells for 48 h (11), sonicated, and stored frozen at -70°C as crude virus stocks in a balanced salt solution containing 0.1% bovine serum albumin. Infection of monkey kidney cells with these viruses resulted in the production of polypeptides that were immunoprecipitable with anti-PR8 antisera and which comigrated with authentic influenza virus proteins in sodium dodecyl sulfate-polyacrylamide gels. Although two proteins are synthesized during influenza virus infection from the gene used to produce NS1-VAC (NS1 and NS2), only NS1 was produced following NS1-VAC infection. During influenza virus infections, NS2 is produced from the mRNA created in the nucleus by splicing of the full-length, plus-stranded RNA. The absence of NS2 during NS1-VAC infection is probably related to the cytoplasmic nature of VAC virus infections.

Medium. Iscoves modified Dulbecco modified Eagle medium (IMDM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) was used for all in vitro cultures.

Cells. All cells were maintained in a humidified air-CO₂ atmosphere at 37°C . P815 cells (a DBA/2 mastocytoma cell line) and MC57G cells (a methylcholanthrene-induced tumor cell line derived from C57BL/6 mice) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. L929 cells (derived from C3H mice) and (K $\times$ D)F₁ cells were maintained in IMDM supplemented with 10% fetal bovine serum. (K $\times$ D)F₁ cells were produced from (DBA/2 $\times$ CBA/J)F₁ mice by the addition of 10^8 PFU of simian virus 40 to primary lung cultures. Clones derived under limiting dilution conditions were screened for class I MHC expression by flow cytometry following indirect immunofluorescence staining with monoclonal antibodies. One clone expressing high levels of class I molecules from both parents

was expanded in bulk culture and used as a target cell in CTL assays.

Immunization. Mice were immunized intravenously with 1×10^8 to 3×10^8 PFU of VAC virus or the VAC virus recombinants. Mice were immunized intraperitoneally with 100 to 300 hemagglutinating units of PR8.

Secondary cultures. Splenocytes from immunized mice were used as in vitro responder cells 2 or more weeks following in vivo priming. Stimulator cells for in vitro secondary responses were obtained from autologous splenocyte populations. Stimulator cells (25×10^6 to 50×10^6) were infected with 2×10^9 egg infectious doses of influenza virus or with 3×10^8 PFU of VAC virus. Secondary cultures of 3×10^7 stimulator cells and 6×10^7 responder cells were incubated in 45 ml of IMDM at 37°C in a 10% CO₂ environment. After 6 days the effectors were harvested and assayed for specific cytotoxicity.

Cytotoxicity assay. Cytotoxicity was assayed by using a ⁵¹Cr-release assay. Targets were prepared by infecting 3×10^6 P815 mastocytoma cells with 3×10^9 egg infectious doses of influenza virus or 3×10^8 PFU of VAC virus. After 1 h of incubation at 37°C , targets were washed and incubated for 6 h at 37°C in 10 ml of IMDM before ⁵¹Cr labeling. After incubation with 250 μCi of Na⁵¹CrO₄, cells were washed twice and diluted to 10^5 cells per ml in IMDM, and 100 μl was added to each well of a 96-well, round-bottom microtiter plate. Effector cells, also in 100 μl of IMDM, were added at ratios of 20:1, 7:1, 2:1, and 1:1. After 4 h of incubation at 37°C in air-CO₂ (90%:10%), 100 μl of supernatant was harvested to determine the concentration of ⁵¹Cr released. All samples were counted in a rackgamma counter (LKB Instruments, Inc., Rockville, Md.), and the percent specific release was calculated as follows: [(experimental release - spontaneous release)/(total release - spontaneous release)] $\times 100$. All assays were performed in triplicate.

RESULTS

Recognition of polymerases and NS1 by influenza virus-specific CTLs. BALB/c (*H-2^d*) recognition of polymerases and NS1 was examined in a ⁵¹Cr-release assay with P815 (*H-2^d*) cells infected with recombinant VAC viruses containing cloned influenza A virus genes (Table 1). To generate cross-reactive CTL populations, splenocytes derived from PR8-primed mice were stimulated in vitro with heterotypic influenza A viruses (JAP or HK). Both of these cross-reactive CTL populations lysed P815 cells that were infected with NP-VAC (included as a positive control); NS1-VAC; PB2-VAC; and, at lower levels, PA-VAC. PB1-VAC-

TABLE 2. Ability of PB2-VAC to prime for secondary in vitro BALB/c influenza virus-specific CTL responses

P815 cells	Percent specific ⁵¹ Cr release from:					
	PB2-VAC/PR8 ^a		PB2-VAC/VAC ^a		VAC/PR8 ^a	
	7:1	2:1	2:1	1:1	2:1	1:1
PR8	48	23	19	8	10	8
PB1-VAC	6	1	93	77	11	4
PB2-VAC	51	26	92	75	8	3
NS1-VAC	6	3	90	47	8	2
NP-VAC	3	1	92	60	8	3
VAC	5	9	92	66	9	12

^a The first strain designation is primary in vivo stimulation; the second strain designation is secondary in vitro stimulation. Cytotoxic assays were performed with BALB/c (*H-2^d*) splenocytes at the indicated effector:target ratios.

TABLE 3. Recognition of influenza virus polymerases and NS1 by CTLs from C57BL/6 mice

MC57G (<i>H-2^b</i>) cell	Percent specific ⁵¹ Cr release from:					
	PR8/JAP ^a splenocytes ^b			NT60/JAP ^a splenocytes ^b		
	7:1	2:1	1:1	7:1	2:1	1:1
PR8	43	32	19	31	15	8
PB1-VAC	14	6	1	17	7	2
PB2-VAC	24	15	7	18	6	2
PA-VAC	47	37	27	38	21	10
NS1-VAC	15	6	3	15	8	4
NP-VAC	45	36	20	31	21	8
VAC	12	6	3	13	8	4

^a The first strain designation is primary in vivo stimulation; the second strain designation is secondary in vitro stimulation.

^b Cytotoxic assays were performed with C57BL/6 (*H-2^b*) splenocytes at the indicated effector:target ratios.

infected cells were lysed at low levels by JAP-restimulated cultures and were not lysed significantly above control levels by HK-restimulated CTLs. Levels of CTL recognition of PB1-VAC and PA-VAC-infected cells were found to vary in a large number of additional experiments (data not shown). In contrast, NP-VAC-, NS1-VAC-, and PB2-VAC-infected P815 cells were consistently recognized by cross-reactive CTL populations.

Priming for an influenza virus-specific CTL response with recombinant VAC viruses. We have previously found that inoculation of mice with recombinant VAC viruses containing cloned influenza or vesicular stomatitis virus genes can prime splenocytes for secondary in vitro responses specific for influenza or vesicular stomatitis viruses, respectively (2, 3, 40, 41). In vitro PR8-restimulated splenocytes derived from PB2-VAC-primed mice specifically lysed PR8-infected P815 cells (Table 2). The specificity of priming was demonstrated by the lack of cytotoxicity in splenocyte cultures derived from VAC virus-primed mice. While CTLs derived from PB2-VAC-primed mice efficiently lysed PB2-VAC-infected cells, they did not recognize cells infected with NS1-VAC or NP-VAC. PB2-VAC primed splenocytes restimulated with VAC lysed all VAC virus- and recombinant VAC virus-infected targets but did not lyse PR8-infected cells. This extends our previous findings (3, 40) that splenocytes derived from recombinant VAC virus-primed mice are only primed for a CTL response against VAC virus and the foreign antigen encoded by the recombinant. Similar

TABLE 4. Recognition of influenza virus polymerases and NS1 by CTLs from CBA/J mice

L929 (<i>H-2^k</i>) cell	Percent specific ⁵¹ Cr release from:					
	PR8/JAP ^a splenocytes ^b			PR8/NT60 ^a splenocytes ^b		
	7:1	2:1	1:1	7:1	2:1	1:1
PR8	83	52	16	99	58	27
PB1-VAC	94	57	24	93	49	16
PB2-VAC	4	0	0	7	2	1
PA-VAC	7	2	1	9	3	1
NS1-VAC	51	26	5	61	42	13
NP-VAC	93	53	21	96	55	19
VAC	13	4	1	10	3	1

^a The first strain designation is primary in vivo stimulation; the second strain designation is secondary in vitro stimulation.

^b Cytotoxic assays were performed with CBA/J (*H-2^k*) splenocytes at the indicated effector:target ratios.

TABLE 5. CTL recognition of PB1 and PB2 cosegregates with the MHC

Target cell	Percent specific ⁵¹ Cr release from the following PR8/HK ^a splenocytes ^b :							
	BALB/c (<i>H-2^d</i>)		B10.D2 (<i>H-2^d</i>)		BALB/K (<i>H-2^k</i>)		B10.Br (<i>H-2^k</i>)	
	7:1	2:1	7:1	2:1	7:1	2:1	7:1	2:1
P815 (<i>H-2^d</i>)								
PB1-VAC	16	8	16	7				
PB2-VAC	83	61	75	49				
NP-VAC	96	97	99	93				
VAC	10	6	12	8				
L929 (<i>H-2^k</i>)								
PB1-VAC					90	73	85	56
PB2-VAC					4	0	13	7
NP-VAC					76	53	77	58
VAC					5	2	10	6
(K × D) _{F1} (<i>H-2^{k×d}</i>)								
PB1-VAC	10	4	10	4	61	54	66	49
PB2-VAC	50	25	41	21	2	3	13	5
NP-VAC	59	51	56	49	64	48	63	47
VAC	16	4	7	3	4	0	14	6

^a Strain PR8 was used for primary in vivo stimulation; strain HK was used for secondary in vitro stimulation.

^b Cytotoxic were performed with splenocytes at the indicated effector:target ratios.

findings were obtained with splenocytes derived from PA-VAC-inoculated BALB/c mice, with the exception that the priming effect was less consistently observed (data not shown).

Recognition of polymerases and NS1 by CTLs derived from CBA/J and C57BL/6 mice. The differential recognition of viral gene products by BALB/c CTLs was further examined by using cross-reactive CTL populations derived from other mouse strains. Consistent with results of a previous report (8), C57BL/6 (*H-2^b*) anti-influenza virus CTLs exhibited relatively low levels of activity against PR8-infected MC57G (*H-2^b*) cells (Table 3). Sufficient CTL activity was present, however, to determine that the pattern of recognition of recombinant VAC virus-infected cells differed from that of BALB/c CTLs. Significant levels of specific lysis were only consistently obtained with NP-VAC- and PA-VAC-infected cells (Table 4). A third pattern of recognition was found with cross-reactive CTLs derived from CBA/J (*H-2^k*) mice. In this case NP-VAC-, PB1-VAC-, and NS1-VAC-infected L929 cells (*H-2^k*) were lysed at high levels, while cells infected with PB2-VAC or PA-VAC were not lysed above control levels (Table 4).

Differential recognition of viral antigens segregates with the MHC. The differential recognition of PB2-VAC- and PB1-VAC-infected cells by BALB/c and CBA/J CTLs was further examined by using cross-reactive CTL populations derived from congenic mice. Polymerase recognition was found to segregate with the MHC haplotype of the responding strain. BALB/c (*H-2^d*) and B10.D2 (*H-2^d*) mice demonstrated high levels of cytotoxic activity against PB2-VAC-infected P815 cells and did not lyse PB1-VAC-infected cells above control values (Table 5). The inverse pattern of recognition was observed on recombinant VAC virus-infected L929 cells with CTLs derived from *H-2^k* congenic mice (BALB/K and B10.Br) (Table 5).

To determine whether these results were due to differential expression of PB1 and PB2 in the target cells used to

assess CTL activity, the same CTL populations were tested against a simian virus 40-transformed cell line derived from (C3H $\times$ DBA/2) F_1 ($H-2^k \times d$) mice. As described above, lytic activity against PB2-VAC-infected cells cosegregated with the $H-2^d$ haplotype, while lytic activity against PB1-VAC-infected cells segregated with the $H-2^k$ haplotype (Table 5). Based on these results we conclude that CTL responsiveness to PB1 and PB2 is MHC controlled.

DISCUSSION

In a previous study we examined the ability of strain-specific cloned CTL cell lines to lyse target cells infected with PR8 $\times$ HK recombinant viruses (1). To our surprise the cytotoxic activity of two of these lines was found to cosegregate with the PR8 gene encoding PB2. At the time we could not distinguish whether these CTL recognized PB2 itself, PB2 induced modifications in cellular (non-MHC) antigens, or PB2 induced modifications in other viral antigens. Based on the present findings the last possibility can be eliminated. Recent findings that the expression of nonfunctional fragments of other viral antigens (NP and HA) sensitizes targets for CTL lysis (33, 35, 38) argues strongly against the second possibility. Thus, it is likely that CTLs recognize PB2 itself or an antigenically active fragment of PB2.

In addition to PB2, we have shown that PA, PB1, and NS1 are recognized by cross-reactive CTLs. The cross-reactive recognition of these proteins by CTLs is consistent with the high degree of amino acid homology between polymerases and NS1 proteins derived from heterotypic influenza viruses (28). Taken together with results from studies of CTL recognition of other influenza virus and vesicular stomatitis virus antigens (2, 3, 6, 34, 40, 41), it appears that in general, CTL recognition of viral proteins parallels the degree of amino acid homology between related proteins. CTL recognition of these internal proteins is not totally cross-reactive, however, because the anti-PB2 clones we originally described were strain specific (1) and strain-specific CTL recognition has been observed for the similarly highly conserved NP (36, 37).

Our demonstration that NS1 is involved in the anti-influenza virus CTL response indicates, for the first time, that CTLs are able to recognize a nonstructural viral antigen that is synthesized during a lytic infection. This finding is not entirely unprecedented, however, because it is known that CTL recognize simian virus 40 T antigen, a nonstructural virus-encoded protein which is present in transformed and abortively infected murine cells (14, 24, 32). The present findings add to the mounting evidence that nonintegral membrane proteins play a critical role in anti-viral CTL response. The fact that CTL recognition of the polymerases is in some cases equal to or better than CTL recognition of NS1 or NP, which are synthesized in far greater quantities, indicates that CTL recognition of internal proteins is relatively independent of the total cell antigen concentration. CTL recognition of cells expressing amounts of cloned viral gene products that are nondetectable by antibody binding is consistent with this view (25, 33).

The pathway by which internal antigens are transported to the cell surface is uncertain. It has been shown that target cells expressing cloned genes encoding as little as 130 residues of NP are recognized by CTLs (33). Additionally, it has been found that target cells can be sensitized for lysis by the addition of synthetic peptides containing as few as 14 amino acids of the NP sequence (35). These findings demonstrate that at least a portion of antiviral CTLs recognize

sequential determinants. They also strongly suggest that the transport of internal proteins to the cell surface is independent of their function. It remains uncertain, however, whether fragmentation of viral antigens is required for CTL recognition.

In this study we have found that CTL responsiveness to NS1 and the polymerases varies greatly between mouse strains and have shown that for at least two of these proteins (PB1 and PB2) responsiveness segregates with the MHC. It is not possible to distinguish whether low responsiveness of MHC reflects the failure of viral antigen to properly associate with certain class I molecules or the absence of CTLs that are able to recognize the putative viral antigen-class I molecule complex. The phenomenon of low responsiveness to viral antigens in association with certain murine MHC class I alleles has been described for a number of viruses (including influenza virus) (5, 8, 9, 12, 13, 17, 22, 23, 26, 27, 42). This has generally been defined at the level of $H-2^k$ locus versus $H-2^d$ locus responsiveness, but in one at least instance (7) it has been shown that an antiviral response is preferentially restricted in $H-2^d$ mice to the product of the $H-2L^d$ locus and that $H-2^d$ mice which fail to express $H-2L^d$ do not mount a CTL response (7). Human antiviral CTLs have also been found to exhibit preferential recognition of certain class I molecules (20, 29).

The fact that MHC-linked responsiveness could be detected at the level of whole antiviral responses in previous studies seems remarkable when considering the number of viral antigens (for influenza virus, at least six) and MHC-restricting molecules (at least two to three) (15, 31) which could interact to form CTL target structures. This suggests that recognition of individual viral components in the context of any given MHC class I allele occurs infrequently. The present findings regarding CTL recognition of the polymerases support this idea, as do observations that murine CTL responsiveness to NP (23a), NS1, the polymerases, and HA (unpublished data) can be mapped to $H-2^k$ or $H-2^d$ loci. This idea has two important implications. (i) CTL responsiveness to individual viral components constitutes at least part of the evolutionary forces which maintain and expand MHC class I polymorphism; and (ii) to decrease the chance of MHC controlled nonresponsiveness, vaccines designed to elicit CTL immunity should not be based on a single viral component but should include as many components as feasible.

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