RECOGNITION OF CLONED VESICULAR STOMATITIS VIRUS INTERNAL AND EXTERNAL GENE PRODUCTS BY CYTOTOXIC T LYMPHOCYTES

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MHC-restricted CTL are believed to play an important role in the immune response to tumors and virus infections (1, 2). CTL recognize self class I MHC molecules in conjunction with foreign antigens, which are either endogenously synthesized by target cells, or less commonly, exogenously provided in a suitable form (1, 3–6). Recent results (7–10) obtained using eukaryotic expression vectors containing cloned genes of influenza virus (an orthomyxovirus) indicate that, contrary to initial expectations, internal viral proteins, which are expressed on infected cell surfaces in relatively low amounts, and not the abundantly expressed integral membrane glycoproteins serve as the major target structures recognized by antiinfluenza CTL.

To determine whether this finding is unique to orthomyxovirus-specific CTL or represents a general feature of CTL recognition, we have examined CTL recognition of internal and external proteins of vesicular stomatitis virus (VSV)[1] (a rhabdovirus). VSV is similar to influenza virus in that both are negative-stranded RNA viruses that obtain their envelope by budding from the host cell. The viruses differ however in many respects, including (*a*) the surface through which viral budding occurs in polarized epithelial cell lines (VSV and influenza virus bud through basolateral and apical surfaces, respectively); (*b*) the nature of their genomes (influenza virus has a segmented genome); and (*c*) their sites of replication (VSV replication occurs entirely in the cytoplasm, while a critical portion of the influenza virus replication cycle occurs in the nucleus).

Two serotypes of VSV (Indiana [IND] and New Jersey [NJ]) can be distinguished by infectivity neutralization assays. Initial studies of VSV-specific CTL identified two general CTL types: one specific for either VSV_{IND}- or VSV_{NJ}-infected cells (specific), the other able to lyse cells infected with either virus

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[1] *Abbreviations used in this paper:* CTL-II, MHC class II–restricted CTL; FLU-VAC, influenza/vaccinia virus recombinant; G, glycoprotein; HAS, PBS containing NaN_3; IND, VSV-Indiana; N, nucleocapsid protein; NJ, VSV-New Jersey; NP, nucleoprotein; ts, temperature-sensitive; VAC, vaccinia virus; VSV, vesicular stomatitis virus.

(crossreactive) (12). It has generally been considered that both of these CTL populations predominantly, if not solely, recognize the VSV glycoprotein (G), an integral membrane protein that coats the virion surface and is abundantly expressed on virus-infected cells. The possibility that CTL recognize the other viral proteins (nucleocapsid [N], matrix [M], nonstructural [NS], or large [L] proteins), which are located internally in infected cells, has received only minor consideration (13, 14). Additionally, the expression of these proteins on cell surfaces has not been examined.

In this study, we have examined the specificity of anti-VSV CTL using recombinant vaccinia (VAC) viruses containing copies of genes encoding VSV G or N. We have found that: (*a*) anti-VSV recognition of G is almost entirely serotype-specific; (*b*) N can be detected on infected cell surfaces by mAb; (*c*) N serves as a major target antigen for crossreactive anti-VSV CTL.

Materials and Methods

Viruses. Stocks of VSV strains IND and NJ were grown in BHK-21 cells. The construction and characterization of recombinant vaccinia viruses containing the IND G (G_{IND}-VAC) and N genes (N_{IND}-VAC), NJ G (G_{NJ}-VAC) and A/PR/8 nucleoprotein (NP) (FLU-VAC) have been described (15). Vaccinia viruses were grown as previously described (10).

Cells. P815 cells (a DBA/2 [H-2^d] mastocytoma cell line) were maintained in RPMI 1640 medium supplemented with 10% FCS in a humidified air/CO_2 atmosphere at 37°C. L929 (H-2^k) cells expressing cloned H-2L^d (LM-1) and H-2D^d (DM-1) MHC class I genes (16) (generously provided by J. Weis and J. Seidman, Harvard Medical School, Boston, MA) were maintained in Iscove's modified DMEM supplemented with 10% FCS (Iscove's medium) in a humidified air/CO_2 atmosphere at 37°C.

Mice. 6–8-wk-old BALB/c (H-2^d), C57Bl/6 (H-2^b), and CBA/J (H-2^k) mice were obtained from the Jackson Laboratories (Bar Harbor, ME).

Preparation of Target Cells. Target cells were prepared as described previously (10). Briefly, P815 cells were infected with VAC or VSV at multiplicities of 10 and 1, respectively, incubated for 5–7 h at 37°C, and labeled with $Na^{51}CrO_4$ for 1 h at 37°C. L929 cells were prepared identically after their removal from tissue culture flasks by brief treatment with PBS containing 0.025% trypsin.

Preparation of Effector Cells. Splenocyte suspensions were prepared from mice primed 3–8 wk earlier by intravenous injection with 10^7 PFU VAC, or by intraperitoneal inoculation with 10^7 PFU VSV. ~6×10^7 splenocytes were cocultured in 40 ml Iscove's medium for 6 d at 37°C with 3×10^7 autologous splenocytes infected with VSV or VAC at a multiplicity of 10.

Microcytotoxicity Assays. In vitro–stimulated splenocytes in 100 μl of Iscove's medium were added at various ratios to round-bottom, 96-well polystyrene plates containing 10^4 target cells in 100 μl Iscove's medium. After 4 h of incubation at 37°C, 100 μl of the supernatant was removed and the concentration of released $^{51}CrO_4$ determined with a gamma counter. Data are expressed as percent specific release defined as: [(experimental cpm) − (spontaneous [no splenocytes] cpm)]/[(total [detergent] cpm) − (spontaneous cpm)]. Spontaneous release values were always <25% of total release values.

Monoclonal Antibodies. mAb specific for VSV antigens were produced and characterized as previously described (17). The specificity of N-specific antibodies was determined by immunoprecipitation.

Immunofluorescence. 8 h after infection with VSV or VAC, P815 cells were pelleted by centrifugation and suspended at a concentration of 3×10^7 cells/ml in PBS containing 0.04% NaN_3 and 1% BSA (HAS-BSA). 25-μl cell suspensions were then added to100 μl of HAS-BSA containing hybridoma ascites fluids diluted 10^{-3}–10^{-4}. After 1.5 h incubation at 4°C, cells were washed three times with HAS-BSA and suspended in 25 μl HAS-BSA

TABLE I

Cytofluorographic Analysis of VSV Antigens on Infected Cell Surfaces

Cells	Anti-G		Anti-N_{IND}		Anti-N_{NJ}	
	Percent positive*	Intensity‡	Percent positive	Intensity	Percent positive	Intensity
VSV_{IND}	78	143	25	59	—	—
N_{IND}-VAC	—	—	25	24	—	—
G_{IND}-VAC	82	96	—	—	—	—
VSV_{NJ}	84	141	—	—	51	66
G_{NJ}-VAC	82	87	—	—	—	—

Binding of mAb to viable cell surfaces 8 h after infection with VSV or recombinant VAC viruses was detected by indirect immunofluorescence analyzed using a cytofluorograph.

* Percentage of cells binding antibody above background levels obtained using uninfected cells (for VSV-infected cells), or FLU-VAC–infected cells (for cells infected with VAC recombinant viruses).

‡ Average relative fluorescent intensity.

containing fluorescein-conjugated rabbit anti–mouse Ig (Dako Corp., Santa Barbara, CA) diluted 1:50. After 1.5 h at 4°C, cells were washed four times with HAS-BSA and either examined in a Leitz photomicroscope equipped with standard epifluorescence optics or fixed for subsequent cytofluorograph analysis. Cells were fixed by 10-min incubation at room temperature in 50 μl PBS containing 3% paraformaldehyde. After addition of 100 μl 0.15 M glycine, cells were pelleted, washed with HAS-BSA and stored overnight at 4°C. Cells were then suspended in 400 μl PBS, and the surface fluorescence of viable cells (before fixation) was quantitated using an Ortho cytofluorograph. Cells that were nonviable at the time of fixation were excluded from cytofluorographic analysis based on their light scattering properties (control experiments established that fixation did not alter the ability of the cytofluorograph to distinguish viable from nonviable cells).

Results

Expression of Viral Antigens on Infected Cell Surfaces. The expression of VSV gene products in P815 cells (a murine cell line used as a CTL target cell) infected with VSV or vaccinia virus was examined by flow cytometry after indirect immunofluorescence staining using anti-VSV mAb. These data are summarized in Table I. Using an mAb that cross-reacts between G_{NJ} and G_{IND}, a large percentage of cells infected with VSV_{NJ} or VSV_{IND} was heavily stained. A similarly large percentage of cells infected with either G_{IND}-VAC or G_{NJ}-VAC was stained, although lower quantities of G were detected. The specificity of binding was shown by the failure of this antibody to bind cells infected with a recombinant VAC virus containing the influenza virus NP gene (FLU-VAC), and by the failure of anti–influenza virus mAb to bind cells infected with VSV or VAC recombinants containing VSV genes (not shown).

mAb specific for N_{NJ} or N_{IND} were used to examine the expression of N on infected cell surfaces. ~50% of cells infected with VSV_{NJ} showed significant binding of the anti-N mAb. 25% of cells infected with VSV_{IND} or N_{IND}-VAC showed binding above background leels, with greater amounts of N being detected on VSV_{IND}-infected cells. Antibody binding specificity was demonstrated as above (not shown). The expression of N on infected cell surfaces was confirmed by direct examination of stained cells, where aggregates of stain were seen to be distributed on the surface of viable cells infected with either VSV or N_{IND}-VAC (not shown).

TABLE II

Recognition of P815 Cells Expressing Isolated VSV Antigens by VSV-specific CTL

Virus-infected P815 Cells	Percent specific ^{51}Cr release							
	VSV_{IND}* VSV_{IND}‡		VSV_{NJ} VSV_{NJ}		VSV_{IND} VSV_{NJ}		VSV_{NJ} VSV_{IND}	
	10:1§	3:1	10:1	3:1	10:1	3:1	10:1	3:1
VSV_{IND}	67	47	47	32	63	47	54	31
VSV_{NJ}	44	26	49	31	52	33	43	23
G_{IND}-VAC	37	16	13	7	12	7	12	3
G_{NJ}-VAC	15	5	69	50	14	6	11	3
N_{IND}-VAC	72	35	57	27	77	49	61	23
FLU-VAC	13	6	13	8	11	6	13	3
Uninfected	16	5	14	7	14	6	13	5

* Primary in vivo stimulation.
‡ Secondary in vitro stimulation.
§ Cytotoxicity assays were performed using BALB/c splenocytes at the E/T ratios indicated.

Recognition of Target Cells Expressing Cloned VSV Antigens by Anti-VSV CTL. Splenocytes derived from BALB/c mice immunized with either VSV_{IND} or VSV_{NJ} and stimulated in vitro with autologous splenocytes infected with VSV_{IND} or VSV_{NJ} were tested for their cytotoxic activity against uninfected P815 cells, or cells infected with either VSV or recombinant VAC viruses (Table II). CTL capable of lysing cells infected with either VSV_{NJ} or VSV_{IND} were present in each of the populations tested. The MHC-restricted nature of target cell recognition was shown by the failure of these splenocytes to lyse VSV infected murine cells expressing other MHC haplotypes (L929 [H-2^k], MC57G [H-2^b]), which were lysed by histocompatible CTL (not shown).

Cells infected with G-VAC recombinants were specifically lysed by splenocytes primed and stimulated with viruses containing the homologous G. The higher levels of lysis of cells infected with G_{NJ}-VAC relative to lysis of G_{IND}-VAC was also observed in other experiments, and is probably due to differences in the effector populations and not to levels of G expression on recombinant VAC-infected cells, since equivalent levels of G were present on the targets (Table I). Neither target expressing cloned G was lysed above control levels by the cross-reactive CTL populations used in this experiment (either splenocytes primed with one serotype and in vitro stimulated with the other serotype, or splenocytes primed and stimulated with a serotype containing the heterologous G), although low levels of crossreactive lysis of G_{NJ}-VAC infected cells were sporadically observed in other experiments (not shown). The failure of cross reactive CTL to recognize G-VAC–infected cells was not due to interference from processes related to the VAC infection, since cells coinfected with G_{NJ}-VAC and VSV_{NJ} or G_{IND}-VAC and VSV_{IND} were efficiently lysed by crossreactive CTL (not shown).

In contrast to the strain-specific recognition of G, N_{IND}-VAC–infected cells were lysed at high levels by all four anti-VSV CTL populations, a finding that was consistently repeated in a number of additional experiments. The specificity of CTL recognition of N_{IND}-VAC-infected cells is indicated by two findings. First, BALB/c CTL specific for G (Table III) or influenza virus (not shown) failed to lyse these cells. Second, N_{IND}-VAC–infected cells were not lysed by anti-

TABLE III

Ability of Recombinant VAC Viruses Containing VSV Genes to Prime for Secondary In Vitro VSV-specific CTL Response

P815 cells	Percent specific ^{51}Cr release							
	G_{NJ}-VAC* VSV_{IND}‡		G_{NJ}-VAC VSV_{NJ}		N_{IND}-VAC VSV_{IND}		N_{IND}-VAC VSV_{NJ}	
	14:1§	4:1	14:1	4:1	14:1	4:1	50:1	4:1
VSV_{IND}	19	6	31	9	83	53	74	47
VSV_{NJ}	17	5	79	44	96	62	69	44
G_{NJ}-VAC	13	3	57	26	9	2	6	3
G_{IND}-VAC	13	3	23	7	4	2	3	1
N_{IND}-VAC	10	3	21	5	79	42	62	26
FLU-VAC	9	2	18	3	7	1	7	1
Uninfected	6	1	15	4	6	0	2	1

* Primary stimulation.
‡ Secondary stimulation.
§ Cytotoxicity assays were performed using BALB/c splenocytes at E/T ratios indicated.

VSV CTL derived from MHC-incompatible mice (CBA/J [H-2^k] or C57Bl/6 [H-2^b]) (not shown).

Priming of Anti-VSV CTL by VAC Recombinant Viruses. We have previously shown (8, 10, 18) that inoculation of mice with recombinant VAC viruses primes their splenocytes for a secondary in vitro response. Inoculation of BALB/c mice with G_{IND}-VAC only primed splenocytes for a weak response upon in vitro challenge with VSV_{IND}-infected cells in two of four experiments, and failed in all four experiments to prime for a response to VSV_{NJ} (not shown). In the same experiments, inoculation with G_{NJ}-VAC consistently primed for a vigorous secondary response upon stimulation with VSV_{NJ}, and occasionally primed for a weak secondary response upon stimulation with VSV_{IND}. Data from one representative experiment are shown in Table III. VSV_{NJ}-stimulated splenocytes derived from G_{NJ}-VAC mice efficiently lysed VSV_{NJ} or G_{NJ}-VAC–infected cells. These cells showed weak cytotoxic activity against VSV_{IND}-infected cells, and failed to lyse G_{IND}-VAC–infected cells above control values. VSV_{IND} stimulation of the same splenocytes generated low levels of crossreactive anti-VSV CTL activity. Taken together with the low levels of lysis of G-VAC–infected cells by crossreactive anti-VSV CTL described above, these data indicate that recognition of G by BALB/c splenocytes stimulated in vitro secondarily is almost entirely serotype specific.

Inoculation of mice with N_{IND}-VAC-primed splenocytes for a vigorous CTL response upon stimulation with either VSV_{NJ} of VSV_{IND}. Both of these populations lysed VSV_{NJ}- and VSV_{IND}-infected cells with equal efficiency, and as expected, lysed N_{IND}-VAC–infected cells. Note also that these populations did not lyse G_{IND}-VAC- or G_{NJ}-VAC–infected cells above control values. Similarly, CTL primed by G_{NJ}-VAC did not lyse N_{IND}-VAC–infected cells above control values. Along with similar findings we have made using VAC recombinants containing cloned influenza virus genes (manuscript in preparation), these data indicate that the inoculation of mice with recombinant VAC viruses only primes

TABLE IV

N Is Recognized in Conjunction with the L^d Molecule

Virus	Transfected class I gene	Percent specific ^{51}Cr release			
		VSV_{IND}* VSV_{NJ}‡		N_{IND}-VAC VSV_{IND}	
		30:1§	10:1	30:1	10:1
VSV_{IND}	L^d	53	33	45	22
N_{IND}-VAC	L^d	53	26	34	17
FLU-VAC	L^d	13	5	4	2
Uninfected	L^d	7	3	1	0
VSV_{IND}	D^d	4	0	0	0
N_{IND}-VAC	D^d	7	3	3	0
FLU-VAC	D^d	6	2	1	0
Uninfected	D^d	7	3	3	2

* Primary stimulation.
‡ Secondary stimulation.
§ Cytotoxicity assays were performed using BALB/c (H-2^d) splenocytes and L929 (H-2^k) target cells at E/T ratios indicated.

their splenocytes for a secondary in vitro response to VAC virus and the foreign antigen encoded by the recombinant.

N Is Recognized in Association with L^d by Anti-VSV CTL. The data presented above clearly indicate that cells expressing N are efficiently lysed by CTL. The MHC-restricted nature of N-specific CTL was further examined using L929 cells (H-2^k) transfected with cloned D^d and L^d genes derived from BALB/c (H-2^d) mice (termed DM-1 and LM-1, respectively [Table IV]). VSV-infected LM-1 cells were specifically lysed by both secondary CTL induced by priming and in vitro stimulation with VSV, and by a N-specific CTL population induced by N_{IND}-VAC priming and VSV_{IND} in vitro stimulation. Additionally, N_{IND}-VAC–infected cells were specifically lysed by both CTL populations. In contrast, DM-1 cells were not lysed by VSV-specific H-2^d-restricted CTL. The failure of VSV-specific CTL to recognize DM-1 cells is consistent with the conclusions of Ciavarra and coworkers (19, 20) that VSV-specific CTL are solely restricted to L^d in the H-2^d haplotype. However, our finding may also be related to low levels of D^d expression on DM-1 cells. Indirect immunofluorescence performed using anti-D^d mAb followed by flow cytometry indicated that, although D^d was expressed on the surface of at least 65% of these cells, the intensity of staining was low relative to that normally seen using P815 cells (not shown). Additionally, while VAC-infected DM-1 cells were specifically recognized by H-2^d restricted VAC-specific CTL, levels of lysis were always lower than those observed using LM-1 cells (not shown).

Discussion

We have found that N represents a major target antigen for VSV-specific CTL produced by secondary in vitro stimulation. The failure of earlier investigators (13, 14) to detect evidence for a major non–G specific CTL population using temperature-sensitive (ts) VSV mutants is possibly explained by the fact that primary CTL populations were used in these studies. Two findings are relevant

to this possibility. First, a number of studies (14, 21) have found that primary anti-VSV CTL populations are often predominantly serotype-specific, while all the anti-VSV CTL populations we have used are highly crossreactive. Second, our present results indicate that anti-G CTL are predominantly serotype-specific. Thus it is possible that the CTL populations used in these ts studies were largely G-specific. Although further experiments are needed to determine the dependence of anti-VSV CTL specificity on the mode of stimulation, it should be noted that Pala and Askonas (22) have shown that the H-2 restriction of antiinfluenza CTL depends on the site of infection, and perhaps ultimately on the cell type that presents antigen to the CTL.

Our finding that G is only poorly recognized by crossreactive anti-VSV CTL is consistent with the findings of Sethi and Brandis (21). It will be necessary to examine CTL recognition of G at the clonal level to determine whether the low level of crossreactive recognition of G reflects a low frequency of G-specific crossreactive CTL able to efficiently lyse target cells, or inefficient recognition of G by higher-frequency crossreactive CTL. In any event, the serotype-specific recognition of G by CTL is strikingly similar to recognition of serotypically distant influenza hemagglutinin molecules by influenza virus A–specific CTL (7–9, 18).

A number of recent reports (23–26) have described specific lysis of virus-infected cells by T cells that recognize antigen in conjunction with MHC class II molecules (CTL-II). Since the P815 target cell line used in the present study is not known to express class II MHC molecules, it is almost certain that we have assayed exclusively class I–restricted CTL. Furthermore, using L cells expressing cloned class I genes, we have unequivocally shown class I–restricted CTL recognition of N. It is important to distinguish between class I– and class II–restricted CTL, since these cells have different requirements for stimulation and target cell recognition. CTL-II lyse targets exposed to inactivated virus or isolated foreign antigens (26). In contrast, class I–restricted CTL only lyse cells expressing endogenously produced foreign antigens or foreign antigens artificially fused into the plasma membrane (1, 3, 4). Only exceptionally can target cells be lysed by addition of foreign antigens without known fusion activity (5, 6).

It has generally been assumed that CTL recognize native antigen present on the external surface of the plasma membrane. While this may pertain to some antigens (integral membrane proteins such as G), it is uncertain whether CTL recognize native or processed forms of internal antigens such as N. In addition to N, a number of other internal virally encoded proteins are now known to be recognized by CTL. These proteins include five influenza virus proteins (NP [9, 10], NS1 [manuscript in preparation], three polymerases [manuscript in preparation]), SV40 T antigen (27–29), and reovirus $\sigma 1$ protein (5). Where it has been possible to examine antigen expression on the cell surface using mAb (N, NP [30, 31], T [32, 33] $\sigma 1$ [5]), all have been detected on the surface of at least some cells expressing these proteins. The fact that a number of the antibodies used do not bind denatured forms of the antigen supports the idea that CTL recognize native forms of internal antigens (33, and unpublished results). On the other hand, it has been shown that cells expressing truncated forms of NP (31) or T antigen (34, 35) are recognized by CTL, even when it was not possible to

detect the presence of antigenically active fragments in the target cells (31). Based on these findings, Townsend et al. (31) proposed that internal antigens are presented as suitable CTL target structures only after being processed by a cytoplasmic degradative pathway. Alternatively, Sharma et al. (36) have hypothesized that export of nuclear proteins to the cell surface such as T antigen and influenza virus NP occurs by virtue of their interaction with the inner wall of the nuclear membrane. While this could pertain to T antigen, which is present in cells over the course of many cell divisions, it does not explain the cell surface expression and CTL recognition of influenza virus NP, which can occur on a high percentage of cells as rapidly as 2–4 h after infection (30). Furthermore, as VSV N appears to be located exclusively in the cytoplasm (37), nuclear residence does not seem to be an absolute requirement for either the expression of internal nucleic acid binding proteins on the cell surface, or their recognition by CTL.

Summary

It has generally been assumed that most if not all CTL specific for vesicular stomatitis virus (VSV)-infected cells recognize the viral glycoprotein (G), an integral membrane protein abundantly expressed on infected cell surfaces. Using recombinant vaccinia viruses containing copies of cloned VSV genes to examine CTL recognition of VSV, we have confirmed that G is recognized by VSV-specific CTL. More interestingly, however, we have also found that nucleocapsid protein (N), an internal virion protein, can be detected on infected cell surfaces using mAb, and serves as a major target antigen for VSV-specific CTL. In contrast to the highly serotype-specific recognition of G, N is recognized by a major population of CTL able to lyse cells infected with either the Indiana or New Jersey VSV serotypes. Using target cells expressing a cloned MHC class I gene, we could directly show that CTL recognition of N occurs in the context of the MHC L^d molecule.

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