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VESICULAR STOMATITIS ANTIGENS RECOGNIZED BY CYTOTOXIC CELLS: ANALYSIS WITH DETECTIVE INTERFERING PARTICLES AND RECONSTITUTED MEMBRANE VESICLES[1]

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We have investigated whether T cells recognize newly synthesized viral proteins as target antigens on vesicular stomatitis virus- (VSV) infected cells. Target cells were adsorbed at a high multiplicity of infection (MOI) with defective interfering (DI) particles of VSV and assessed for susceptibility to T cell-mediated lysis. Cells adsorbed with DI particles lacking functional RNA were not lysed; however, one DI particle that contained functional RNA did generate target antigens for anti-VSV effector cells. Since this DI particle is capable of translating virus-specific proteins, these data demonstrate an obligatory requirement for viral protein synthesis for the creation of VSV target antigens recognized by cytotoxic T lymphocytes.

In order to directly test whether T cells can recognize a viral antigen present in the mature virion, we prepared reconstituted membrane vesicles containing VSV virion structural proteins and H-2 antigens and asked whether these "mixed" vesicles can trigger secondary anti-VSV effector cells *in vitro*. Our results demonstrate that mixed reconstituted membrane vesicles induce potent anti-VSV cytotoxic cells that are restricted for the *H-2* haplotype derived from the same H-2 antigens present in the lipid bilayer. Anti-VSV responses were not observed when using either reconstituted viral envelopes devoid of H-2 antigens or vesicles containing H-2 antigens but not VSV proteins. Thus, insertion of a structural viral component and H-2 molecule into the same lipid bilayer is sufficient to induce an H-2 restricted anti-VSV cytotoxic response. We suggest that during VSV infection there is a requirement for viral protein synthesis in order to create target antigens for cytotoxic T lymphocytes; whether there is a requirement for a newly synthesized viral component at the induction phase remains to be determined.

Mice infected with virus generate cytotoxic thymus-derived (T) lymphocytes (CTL)[2] that are virus specific (1). The ability of virus-specific CTL to lyse infected targets is restricted by gene products encoded in the *H-2K* and *H-2D* region of the major histocompatibility complex (MHC) (2, 3).

An important question arising from these studies is the nature of the target antigens on the plasma membrane that renders a cell susceptible to T cell attack. It has been suggested that input virions adsorbed on the plasma membrane interact with appropriate self markers to generate H-2 restricted virus-specific determinants. This mechanism is probably operative during Sendai virus infection (4). However, Sendai virus possesses a fusion factor that allows insertion of its viral proteins into the plasma membrane where they are recognized in the context of self H-2. Since most viruses lack a fusion factor, this may represent an unique mechanism not operative during infection with other types of viruses. Consistent with this view are studies with viruses, such as ectromelia and influenza virus, in which virus adsorption does not sensitize a target to lytic attack; this only occurs after viral protein or glycoprotein synthesis (5–7).

Recent studies with influenza virus suggest that viral protein synthesis is not required to trigger T cells despite the absence of a fusion factor in this virus. For example, the addition *in vitro* of purified influenza hemagglutinin (H), in a vesicle-free form, induces anti-influenza cytotoxic effector cells (8, 9). If the induction and effector phases of CTL activities are equivalent, then these studies with influenza H appear to contradict previously mentioned studies done at the target cell level in which the adsorption of influenza virus does not sensitize a cell for immune lysis. However, interpretation of the influenza H data is complicated by the fact that these responses have not been analyzed with F_1 responder cells; thus, H protein may trigger a T helper cell (T_H) that releases a soluble factor that secondarily stimulates CTL precursors (CTL_p) to differentiate into effector cells. This issue has recently been addressed by Henney (10). In short, studies with influenza H demonstrate that primed CTL_p are triggered by a viral antigen that can be found in the mature virion. What is not addressed by these studies is whether this antigen must be newly synthesized and inserted into the plasma membrane to induce a cytotoxic response.

Our studies with vesicular stomatitis virus (VSV), which are in agreement with the recent report of Loh *et al.* (11), demonstrate that membrane vesicles containing H-2 antigens and viral protein induce anti-VSV cytotoxic effector cells that are restricted to the alloantigens incorporated into the vesicle. Since

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[2] Abbreviations in this paper: CTL, cytotoxic thymus-derived lymphocytes; MHC, major histocompatibility complex; H, hemagglutinin antigen; T_H, T helper cell; CTL_p, cytotoxic thymus-derived lymphocyte precursors; VSV, vesicular stomatitis virus; DI particles, defective interfering particles; CML, cell-mediated lympholysis; MOI, multiplicity of infection; VSV_{IND}, Indiana serotype of VSV; VSV_{IND-HR}, heat-resistant variant of VSV; DI-1, defective interfering particle of VSV termed VSIts$^+$ATCC *DI 0.38* (5′, DI-1); DI-2, defective interfering particle of VSV termed VSVts$^+$ATCC *DI 0.54* (5′, DI-2); DI-3, defective interfering particle of VSV termed VSVts$^+$ATCC *DI 0.63* (5′, DI-3); DI-LT, defective interfering particle of VSV termed VSI HR ATCC *DI 0.67* (3′, DI-LT); F-GARIg, fluorescein-conjugated goat anti-rabbit immunoglobulin serum; FACS, fluorescence-activated cell sorter.

these studies were done with F_1 responder cells, these results suggest that viral protein synthesis is not required for induction of anti-VSV cytotoxic responses. It was of interest, therefore, to assess the requirement for viral protein synthesis at the target cell level. For this reason, defective interfering (DI) particles of VSV were adsorbed onto target cells and their susceptibility to T cell-mediated lysis was evaluated. Our results are the subject of this report.

MATERIALS AND METHODS

Mice. Eight to 12-week-old mice of the BALB/c(*H-2*^{d}), C57BL/6(*H-2*^{b}), DBA/2 (*H-2*^{d}), C3B6F$_1$ (C3H×C57BL/6, Ly 1.2, *H-2*^{k}/*H-2*^{b}) and BDF$_1$ (C57BL/6×DBA/2, *H-2*^{b}/*H-2*^{d}) strains were obtained either from our breeding colonies or the Jackson Laboratories, Bar Harbor, Maine.

Tumors. The P815 Mastocytoma (*H-2*^{d}) was maintained in ascites by serial passage in DBA/2 mice. The EL4 (*H-2*^{b}) thymoma was maintained in the same way in C57BL/6 mice. P815 cells were also adopted for growth *in vitro* and this line was used whenever P815 target cells were required in the cell-mediated lympholysis (CML) assay.

Virus. Indiana serotype of VSV (VSV$_{IND}$) and a heat-resistant variant of VSV$_{IND}$ (VSV$_{IND-HR}$) were originally obtained from Dr. L. Prevec, McMaster University, Canada. The virus stocks were prepared from five consecutive plaque purifications followed by two additional plaque purifications in cells pretreated with actinomycin D to avoid defective interfering particles (12). DI particles were prepared from high multiplicity of infection (MOI) passages of VSV$_{IND}$ and VSV$_{IND-HR}$. VSV$_{IND}$ induced small DI particles whereas the VSV$_{IND-HR}$ induced large DI particles (13).

By definition, DI particles are defective virus particles that can replicate only in the presence of standard helper virus. Their defect lies in a loss of a portion of viral genome; however, DI particles of VSV contain identical structural proteins as the infectious virions (14). There are two major classes of DI particles in the VSV system. One class of DI particles contain varying amounts of RNA sequences representing the 5′ half of the infectious virion genome; the other class of DI particles represents the 3′ half of the genome (Fig. 1) (15).

DI particles containing RNA representing the 5′ half of the genome are designated as VSIts$^+$ATCC *DI 0.38* (5′, DI-1), VSIts$^+$ATCC *DI 0.54* (5′, DI-2) and VSIts$^+$ATCC *DI 0.63* (5′, DI-3, (16)). These DI particles are incapable of synthesizing any viral specific proteins (17). We will refer to these DI particles as DI-1, DI-2, and DI-3 in this paper.

In contrast, one DI particle, designated VSI HR ATCC *DI 0.67* (3′, DI-LT), which is obtained from a heat-resistant strain ov VSV$_{IND}$ (13), represents the 3′ half of the infectious virus genome and contains functional RNA sequences that produce limited amounts of at least M and G proteins in infected cells without helper virus (17, 18). We will refer to this DI particle as DI-LT in this paper. Nucleic acid hybridization using purified mRNA and labeled DI-RNA (17) together with oligonucleotide finger print analyses (19) render DI genome mapping (Fig. 1).

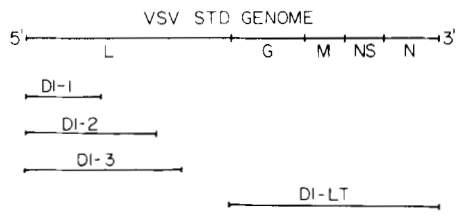

Figure 1. Map of DI particle RNA genomes based on nucleic acid hybridization between messenger RNA's of VSV and DI particle RNA's and on oligonucleotide finger print analysis of DI particle RNA's.

A concentrated stock of VSV$_{IND}$ was prepared by infecting confluent monolayers of R(B77) cells with VSV as previously described (12). After 16 hr of incubation, the supernatants were harvested and centrifuged (600 × G, 20 min) to remove cellular debris. The supernatant was then centrifuged at 80,000 × G for 75 min to pellet infectious VSV. The concentrated virus was resuspended in phosphate-buffered-saline (PBS) and stored at −60°C. This material was used as a source of viral proteins to prepare reconstituted vesicles containing VSV proteins.

A group *IV* mutant of VSV$_{IND}$TSG41 (20) was obtained from D. H. L. Bishop and the tsG41 stock was prepared after three consecutive plaque purification at 33°C in R(B77) cells. This temperature-sensitive mutant of VSV cannot replicate virus at the nonpermissive temperature due to a defect in the function of its nucleoprotein. The titer of the virus at 33°C was 2×10^8 PFU/ml.

Immunizations. BDF$_1$ and BALB/c mice were sensitized against VSV$_{IND}$ by a single i.p. injection of cell-free culture fluid from VSV-infected P815 cells containing 1 to 5×10^8 PFU of VSV. Spleen cells from BALB/c were obtained on day 6 as a source of primary anti-VSV effector cells. Mice primed against alloantigens of P815 were obtained by challenging C3B6F$_1$ mice with a single i.p injection of 30×10^6 viable P815 cells. Approximately 1 month later, splenic lymphocytes from BDF$_1$ and C3B6F$_1$ mice were restimulated *in vitro* to elicit either secondary anti-VSV or H-2^d cytotoxic effector cells, respectively.

Antiserum. A rabbit anti-VSV serum was prepared as previously described (14). The antiserum was rendered virus-specific as follows: P815 cells (5×10^8) were pelleted by centrifugation and then resuspended in 1 ml of antiserum. The serum-cell mixture was incubated for 30 min at 4°C. The cells were again pelleted by centrifugation and the serum was harvested. The adsorption was repeated three additional times, each time with 5×10^8 P815 cells. After the last adsorption, the antiserum was centrifuged at 10,000 × G for 30 min to remove cell debris.

A fluorescein-conjugated goat anti-rabbit immunoglobulin serum (F-GARIg) was obtained from Miles Laboratories, Elkhart, Ind.

Immunofluorescent staining of adsorbed VSV. An indirect (double-antibody) fluorescence assay was used to assess the extent of binding of VSV to P815 cells. All procedures were carried out at 4°C and all antisera were centrifuged at 10,000 × G for 30 min before use. Antisera were used at saturating concentrations as determined by prior titration.

P815 cells (10^6) were pelleted by centrifugation and resuspended in 0.1 ml of VSV$_{IND}$ or various types of DI particles of VSV. The number of virus particles per cell was the same (7×10^3 virions/cell) in all cases. The cell-virus mixtures were incubated for 30 min, washed, and resuspended in 0.1 ml rabbit anti-VSV that was adsorbed four times with P815 cells (see *Antisera*). After a 15-min incubation, the cells were washed in PBS and resuspended in 0.1 ml of F-GARIg diluted 1:3 in PBS. After a 15-min incubation, the cells were washed three times in PBS. The cell pellet was resuspended in 1 ml of isotonic saline and analyzed by the fluorescence activated cell sorter (FACS, Beckton-Dickinson FACS-III).

Fluorescein-labeled cells were exposed to a laser light of 488 nm at an intensity of 300 milliwatts. The fluorescence signal was determined with a photomultiplier potential of 500 volts and a gain setting of 0.5. Ten thousand cells were analyzed to obtain the profiles presented in Figure 2.

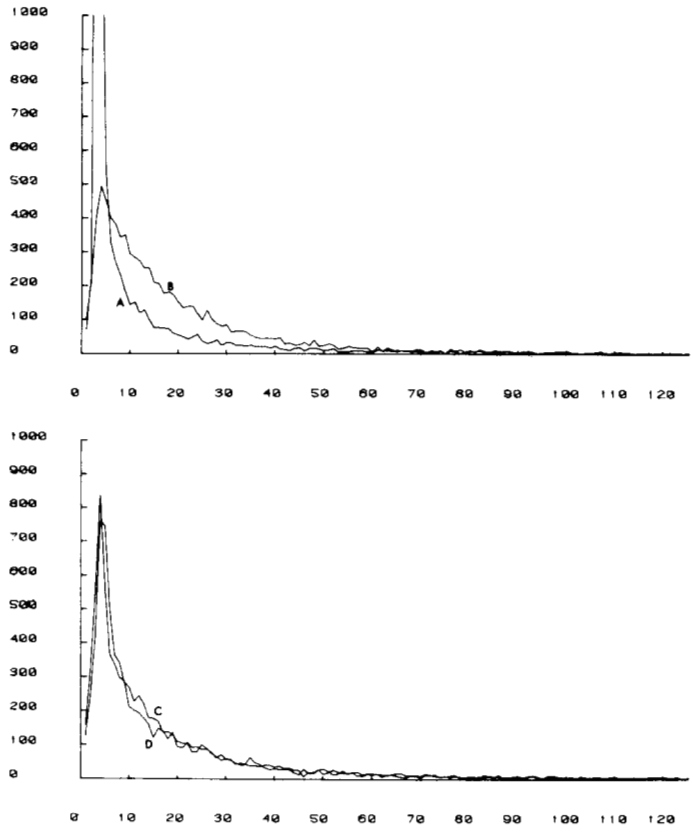

Figure 2. Analysis of the binding properties of VSV_{IND} and DI particles of VSV. All samples of P815 cells were incubated with a rabbit-anti-VSV serum and a F-GARIg as described in *Materials and Methods*. Before antisera treatment, cells were either incubated alone (*A, top panel*) or with one of the following virus preparations (7×10^3 particles/cell); VSV_{IND} (*B, top panel*), DI-3 (*C, bottom panel*), or DI-LT (*D, bottom panel*). Ten thousand cells were analyzed with a FACS and the number cells (*ordinate*) were graphed against fluorescent intensity (*abscissa*) to obtain these profiles.

Generation of secondary cytotoxic effector cells. Spleen cells from mice primed *in vivo* against VSV_{IND} or P815 were restimulated *in vitro* for 5 days with various types of membrane preparations. The culture conditions were as previously described with one exception (21); 20% fetal calf serum (FCS) was added to the culture medium when the immunogen was reconstituted VSV membranes.

Isolation of tumor cell membranes. Membranes from P815 cells were prepared following the procedure of Lemonnier *et al.* (22) with some minor modifications. Tumor cells were disrupted by N_2 cavitation and nuclei and mitochondria were removed by low speed centrifugation (3600 × G, 20 min). Microsomes were obtained from the supernatant by centrifugation at 100,000 × G for 120 min. The pelleted microsomes were resuspended in sterile distilled water and stored at −60°C.

Virus infection. Intact VSV-infected BDF_1 stimulator cells were prepared as follows: Spleen cells were pelleted by centrifugation and resuspended in 0.2 ml VSItsG41. Virus adsorption was allowed to proceed for 1 hr at 37°C. The cells were then resuspended at 1×10^7/ml and incubated (10^6 cells/dish) with VSV_{IND} primed BDF_1 spleen cells at the nonpermissive temperature (39.5°C) for 5 days. Culturing at this temperature allowed the virus-infected BDF_1 stimulator cells to present VSV antigens without allowing the release of infectious virus.

Virus-infected targets were prepared by pelleting 1×10^7 ^{51}Cr-labeled P815 or EL-4 cells and resuspending the tumor cells in 0.2 ml VSV_{IND} (80 PFU/cell). After 45 min at 37°C, 5 ml of supplemented tissue culture medium (21) were added and the cell-virus mixture was incubated an additional hour. The cells were then washed twice to remove unadsorbed virus and resuspended at 10^5/ml.

In order to prepare reconstituted membrane vesicles from VSV_{IND}-infected tumor cells, P815 cells were adsorbed with VSV (20 PFU/cell) for 45 min at 37°C. Tumor cells were resuspended at 2×10^6/ml in supplemented tissue culture medium and incubated an additional 6 hr at 37°C. Microsomes and reconstituted membrane vesicles were prepared from virus infected P815 cells following the procedures outlined for uninfected tumor cells (see below).

CML assay. After 5 days in culture, spleen cells were assessed for cytotoxic T lymphocyte (CTL) activity in a 4 hr ^{51}Cr-release assay as previously described (21). Net isotope release is defined as the isotope released in the presence of immune cells minus isotope released in the presence of normal cells.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* (23) with bovine serum albumin as a standard.

Dissociation and reconstitution of viral envelopes and membrane vesicles. VSV structural proteins and membranes isolated from uninfected and VSV-infected tumor cells were solubilized and reassembled by the method of Finberg *et al.* (24) with minor modifications. Briefly, P815 microsomes and VSV proteins were solubilized in 0.5% sodium deoxycholate (5/1 detergent to protein ratio) for 15 min at 0°C. Detergent insoluble material was pelleted by ultracentrifugation (100,000 × G for 30 min) and the solubilized proteins were used to prepare various types of reconstituted membrane vesicles. Mixed vesicles were prepared by dialyzing P815 membranes and VSV proteins in the same dialysis bag. Vesicles containing either P815 or VSV proteins were obtained by dialyzing these components separately. Dialysis was terminated after >99% of the detergent was eliminated as measured by ^3H-labeled sodium deoxycholate (New England Nuclear, Boston, Mass.). The reconstituted membranes could be frozen (−60°C) and thawed without loss of biologic activity.

RESULTS

Role of viral function in creating VSV target antigens. The nature of the viral antigen on the target cell membrane remains unresolved. Two hypotheses have been proposed to explain *H-2* restricted, virus-specific cytolysis: 1) Input virions adsorbed on the cell surface membrane interact with H-2 antigens to create CML target antigens. 2) Newly synthesized viral proteins inserted into the plasma membrane interact with H-2 antigens to create target antigens for anti-viral effector cells. We have observed, for example, that with high MOI some cytotoxicity was detected against VSV-infected target cells in the presence of pactamycin, an inhibitor of protein synthesis (data not shown). Whether this represents residual host cell protein synthesis that is not inhibited by the pactamycin or the creation of a target antigen on the cell surface that does not involve protein synthesis cannot be ascertained.

To resolve this question we utilized four different types of DI particles of VSV. Three DI particle preparations, DI-1, DI-2, DI-3, derived from VSV_{IND}, represent portions of the 5′ half of the infectious virion RNA genome and, therefore, are unable to be transcribed (17); whereas a fourth DI particle, designated LT, was derived from $VSV_{IND\text{-}HR}$, which represents the 3′ half of the genome. This latter particle is capable of synthesizing partial VSV-specific structural proteins in infected cells (17, 18).

TABLE I

Cytotoxic effect of BALB/c anti-VSV effector cells against target cells exposed to standard VSV or DI particles of VSV

Effector Cell	Virus Preparation	No. Viral Particles/Cell × 10^{4a}	Net ^{51}Cr Release[b]											
			Effector-to-target cell ratio											
			200			100			50			25		
			Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3
Balb/c anti-VSV	Standard	7.5	—[c]	38	34	—	32	31	—	21	17	—	11	10
		0.75	30	33	32	28	30	24	21	18	15	9	8	8
		0.075	24	26	—	24	23	—	15	14	—	8	8	—
	DI-LT	8.1	—	35	29	—	29	24	—	18	14	—	10	7
		0.81	12	34	19	12	28	15	14	21	7	8	9	3
		0.081	17	27	—	19	24	—	13	17	—	7	10	—
	DI-1	11.6	—	5	5	—	3	5	—	2	1	—	1	0
		1.16	1	1	1	0	0	2	0	0	0	0	0	0
		0.116	0	1	—	0	1	—	0	0	—	0	1	—
	DI-2	11.9	—	—	7	—	—	7	—	—	5	—	—	2
		1.19	—	—	2	—	—	3	—	—	2	—	—	1
		0.119	—	—	—	—	—	—	—	—	—	—	—	—
	DI-3	11.3	—	—	9	—	—	7	—	—	5	—	—	3
		1.13	—	—	2	—	—	1	—	—	2	—	—	0
		0.113	—	—	—	—	—	—	—	—	—	—	—	—

[a] Viral particle number was estimated by protein concentration.

[b] Percent ^{51}Cr release from P815 target cells in the presence of nonimmune (control) spleen cells was less than 10%.

[c] —, not done.

When gradient-purified VSV was utilized to infect P815 target cells (Table I), a strong cytotoxic effect was observed. Gradient purified DI-LT particles, which have the ability to synthesize viral proteins, also were able to create target antigens since these target cells were lysed to an extent similar to cells exposed to the gradient-purified infectious standard virus. However, when DI-1, DI-2, and DI-3 particles were utilized no cytotoxic effect was observed. Since the target cells used in this study were assayed against an effector population that was not strongly cytotoxic for VSV_{IND}-infected targets, we restimulated VSV-primed mice *in vitro* to obtain an enriched population of anti-VSV killer cells. Effector cells obtained after restimulation *in vitro* were highly cytolytic for VSV_{IND} and DI-LT targets; again, no lysis of DI-1 and DI-3 target cells was observed (Table II). Since DI-1, DI-2, and DI-3 particles were used at very high particle to cell ratios (10^5) and are able to absorb to the cell surface and enter the cell but cannot synthesize viral proteins by themselves, these data indicate that there is an absolute requirement for viral gene function in order to create antigenic determinants on a VSV-infected target cell. Since gene functions require protein synthesis we conclude that VSV cannot form antigenic determinants on target cells without new virus-specific protein synthesis.

Binding of VSV and DI particles to target cells. In order to demonstrate that DI particles of VSV bind to target cells as well as wild-type virus, P815 cells were adsorbed with either DI-3, DI-LT or VSV_{IND}. After adsorption (30 min, 4°C) the cells were washed one time to remove unadsorbed virions and then incubated with a rabbit anti-VSV serum followed by a F-GARIg. The extent of binding was quantitated by FACS analysis. Figure 2, which graphs the fluorescent intensity (abscissa) against cell number (ordinate), demonstrates that the majority (71%) of the cells were dull or weakly stained (chanels 1 to 10) when incubated with both antisera but not VSV treated. Prior adsorption with VSV_{IND} or with either of the DI particle preparations resulted in a large population of brightly stained cells.

TABLE II

Failure of secondary immune BDF_1 anti-VSV effector cells to detect VSV antigen on cells infected with DI particles of VSV lacking viral gene function

Effector Cell[a]	Virus[b]	Net ^{51}Cr-Release[c] at Effector to Target Ratio of:		
		50	10	5
BDF_1 anti-VSV	VSV_{IND}	55	28	22
	DI-1	0	0	0
	DI-3	1	0	1
	DI-LT	44	26	20

[a] BDF_1 spleen cells from mice sensitized *in vivo* against VSV_{IND} were restimulated *in vitro* for 5 days at 40°C with BDF_1 stimulator cells infected with VSItsG-41.

[b] Labeled target cells were adsorbed with VSV_{IND} (80PFU/cell) or with DI particles of VSV (5×10^5 particles/cell). Following adsorption, cells were incubated and washed as described in *Materials and Methods.*

[c] Values represent means of triplicate determinations.

For example, 71, 67, and 68% of the cells were found in chanels 10 to 120 after adsorption with VSV_{IND}, DI-3, and DI-LT, respectively.

Further, in comparing the fluorescent intensity of P815 cells incubated with either DI-3 or DI-LT particles (bottom panel, Fig. 2) it is evident that both populations have equal fluorescent intensities throughout the profile. Thus, the functional difference between DI-3 and DI-LT cannot be attributed to a difference in their ability to bind to P815 cells. Similar results were obtained in a separate series of experiments by using ^{35}S-methionine-labeled virus. P815 cells incubated with labeled VSV_{IND} or DI-1 particles bound 16 and 17%, respectively, of the input virions (data not shown).

Generation of specific allogeneic CTL response with reconstituted membrane vesicles. Our results with DI particles are compatible with two interpretations; 1) A preformed structural protein that functions as the target antigen cannot interact with

MHC gene products in the cell membrane to generate an immunogenic configuration that the T cell receptor can recognize; this only occurs after penetration and *de novo* viral protein synthesis. The newly synthesized viral structural proteins are then inserted into the plasma membrane where they are recognized in the context of self H-2 antigens. 2) Alternatively, the requisite for viral protein synthesis indicates that target antigen expression requires a metabolic alteration of a component on the cell surface membrane; this could reflect a metabolic alteration of either a host or viral component. If the former interpretation is correct, solubilized viral proteins from mature virions should trigger T lymphocytes when incorporated into a lipid bilayer (reconstituted membrane vesicle) containing H-2 antigens. If the second interpretation is correct, these vesicles will not be immunogenic for T cells.

Since the recognition of viral antigens by CTL requires the recognition of self H-2 antigens (1, 3), the ability of P815 membranes and reconstituted vesicles to elicit a secondary CTL response against H-2^d alloantigens was first evaluated.

Spleen cells from C3B6F_1 mice primed against P815 tumor cells were co-cultured *in vitro* with reconstituted P815 membrane vesicles. The results, presented in Figure 3, demonstrate that P815 reconstituted membrane vesicles induce a potent secondary CTL response. This response is dose dependent and specific for the stimulating H-2 antigens present in the vesicle; thus, no lysis is detected against labeled EL4 targets (Table III).

In contrast with previous reports (22, 25), effector cells could not be elicited *in vitro* when tumor cell membranes were cultured with unprimed spleen cells (data not shown).

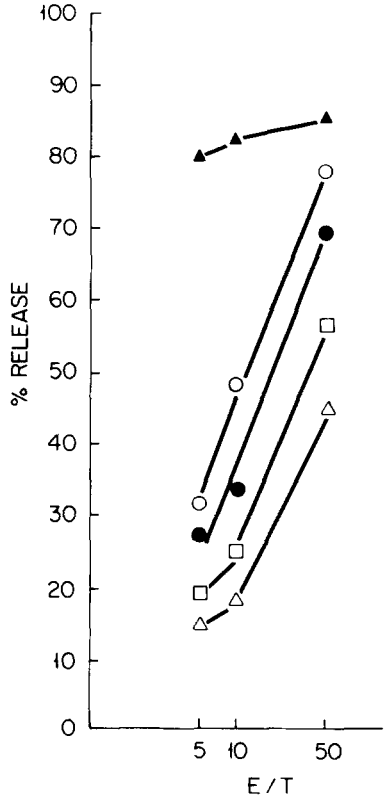

Figure 3. Induction of secondary anti-P815 effector cells by various concentrations of reconstituted P815 membranes. C3B6F_1 mice challenged approximately 6 weeks previously with viable P815 cells were cultured alone (△) *in vitro* or with one of the following immunogens: 1 × 10^6 irradiated Balb/c spleen cells (▲); 8 μg reconstituted membrane (○); 2 μg reconstituted membrane (●); 0.5 μg reconstituted membrane (□). On day 5, the cultures were tested for CTL activity against labeled P815 targets at the indicated effector to target ratio.

TABLE III

Induction of specific anti-P815 cytotoxic effector cells with reconstituted membranes

Immunogen	P815 Target			EL4 Target		
	E/T ratio[a]			E/T ratio		
	50/1	10/1	5/1	50/1	10/1	5/1
	% net release					
Irradiated Balb/c spleen cells	41	58	52	2	1	0
4.0 μg P815 microsome	11	5	0	1	1	0
8.0 μg reconstituted P815 membrane	18	11	2	1	2	0

[a] Effector/target ratio.

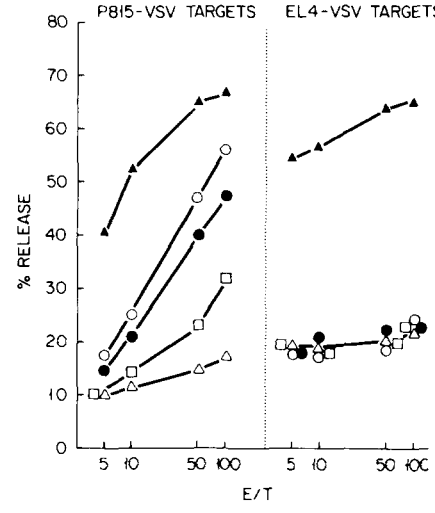

Figure 4. Induction of H-2 restricted anti-VSV cytotoxic activity by reconstituted membrane vesicles. Microsomes obtained from VSV-infected P815 cells were disrupted by sodium deoxycholate and reassembled as described in *Materials and Methods.* BDF_1 responding spleen cells sensitized *in vivo* against VSV were co-cultured with either 3.3 μg (○), 0.8 μg (●), or 0.2 μg (□) of reconstituted P815 membrane. The activity of unstimulated cultures (△), and those cocultured with intact BDF_1 spleen cells infected with VSItsG41 (▲) are included as controls.

Induction of H-2 restricted anti-VSV effector cells by reconstituted membrane vesicles isolated from virus-infected tumor cells. Since our previous data indicated that new viral protein synthesis is a requisite for the generation of target antigens, reconstituted membranes from VSV-infected cells were first tested for their ability to elicit anti-VSV effector cells. Reconstituted membrane vesicles, isolated from P815 tumor cells infected with VSV for 6 to 7 hr, stimulated a potent secondary anti-VSV cytotoxic response in BDF_1 mice (Fig. 4). This response was unequivocally H-2 restricted at the induction stage since no lysis of VSV infected EL4 (H-2^b) targets was observed. It should be pointed out that microsomes isolated from VSV-infected P815 cells elicited a potent anti-VSV cytotoxic response that was not H-2 restricted (data not shown) despite the fact that microsomes, like reconstituted membranes, induced a strong allogeneic anti-P815 cytotoxic response (data not shown). The failure to demonstrate H-2-restricted killing with microsomal membranes probably reflects the presence of infectious virus co-purified with the microsomes; detergent treatment disrupts infectious virions so that virus cannot infect F_1 cells and present viral antigen in the context of H-2^b.

Induction of anti-VSV effector cells with reconstituted membranes containing VSV structural proteins. In order to assess

directly whether a viral component present in the mature virion can trigger anti-VSV cytotoxic responses, P815 membranes and VSV proteins were solubilized in detergent, mixed, and allowed to reassemble into a membrane vesicle. Vesicles containing P815 alloantigens or VSV antigens only were also prepared.

Figure 5 illustrates that reconstituted vesicles expressing P815 alloantigens and VSV proteins in the same membrane elicit a strong anti-VSV cytotoxic response. This response is dose dependent and restricted (Fig. 5) for the alloantigens used to prepare the vesicles. That reconstituted membranes possess biologically active P815 alloantigens is also illustrated by their ability to induce an allogeneic anti-P815 cytotoxic response (Fig. 6). Anti-VSV effector cells were not elicited when primed spleen cells were cultured with vesicles containing either P815 alloantigens or VSV proteins alone (Fig. 5); thus, induction of effector cells requires the presence of H-2 and viral antigens in the same membrane vesicle. It should be noted that in none of our studies have we ever generated anti-VSV responses with membrane vesicles that lacked biologically active H-2 alloantigens (data not shown).

DISCUSSION

The present studies were designed to assess whether recognition of VSV antigens by CTL requires viral protein synthesis. Zinkernagel *et al.* (26) reported that cells adsorbed with DI particles of VSV were not lysed by anti-VSV effector cells. However, these results are difficult to interpret because these particles were UV-treated before adsorption, a procedure that results in reduced binding of VSV to the plasma membrane (data not shown). In our studies, cells infected at high MOI with DI particles were not rendered susceptible to lysis unless the DI particle was capable of translating viral proteins. All DI particles used in our experiments bound as efficiently as wild-type virus to the target cell membrane and possessed intact G protein. Thus, our studies with DI particles of VSV indicate an obligatory requirement for viral protein synthesis in order to create target antigens for CTL.

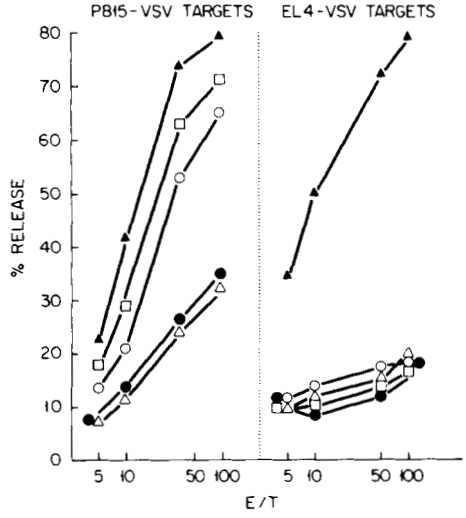

Figure 5. Induction of H-2 restricted anti-VSV effector cells in the absence of viral protein synthesis. Reconstituted membrane vesicles containing P815 membrane and VSV proteins were prepared as described in *Materials and Methods.* Reconstituted P815 membrane vesicles devoid of VSV proteins and reconstituted VSV envelopes were also tested. BDF_1 responding spleen cells sensitized *in vivo* against VSV were cultured either alone (△) or with one of the following immunogens: intact BDF_1 cells infected with VSItsG41 (▲); 6 μg reconstituted membrane vesicles prepared from P815 cells infected with VSV (□); 6.5 μg "mixed" reconstituted membrane vesicles containing approximately 4.5 μg P815 membrane protein and 2 μg VSV protein (○); 2.0 μg reconstituted VSV envelope (●). No anti-VSV activity was generated with reconstituted membranes containing only P815 membrane proteins (data not presented).

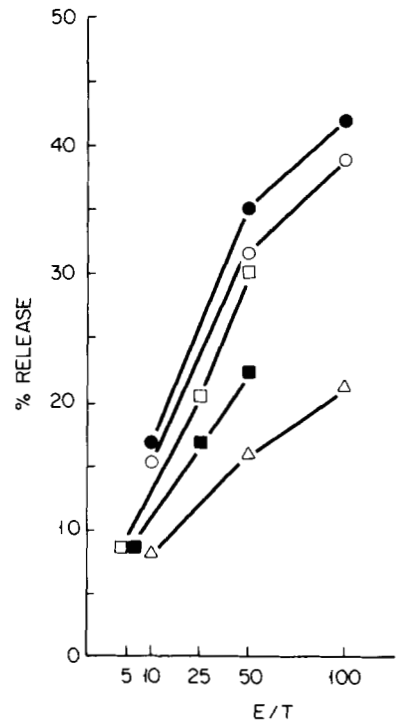

Figure 6. Induction of anti-P815 ($H\text{-}2^d$) cytotoxic effector cells by "mixed" reconstituted membrane vesicles. P815 membrane proteins were solubilized and allowed to reassemble either alone or in the presence of solubilized VSV proteins to create "mixed" vesicles containing H-2 and VSV antigens. Spleen cells from $C3B6F_1$ mice that had survived a live challenge of P815 cells were cultured alone (△) or with 16 μg reconstituted P815 membrane (●). Responder spleen cells were also restimulated with the mixed vesicle at one of the following concentrations: 16 μ (○); 4 μg (□); 1 μg (■). The mixed vesicle contained P815 cell membrane protein and VSV protein at approximately a two to one ratio.

G-protein, the only viral membrane glycoprotein, is the most likely candidate for T cell recognition since temperature sensitive mutants of VSV that cannot express G-protein at the cell surface cannot sensitize cells for immune lysis (26, 27) and antibody against G-protein blocks lysis of VSV-infected cells by anti-VSV killers (27). Similar results have been reported for ectromelia virus in which inhibition of early protein synthesis (or early mRNA transcription) no longer renders cells sensitive to T cell lysis (5). Interestingly, a viral glycoprotein also appears to be involved in T cell recognition of this virus since inhibition of glycosylation (at concentrations that do not significantly inhibit total protein synthesis) prevents the lysis of infected target cells by anti-ectromelia effector cells (28).

The simplest explanation for the failure of large numbers of adsorbed input VSV and poxvirus to sensitize cells to immune lysis is that, unlike Sendai virus, these virions lack a fusion protein or factor. That the mere adsorption of preformed viral components on the plasma membrane is insufficient for CTL recognition is clearly demonstrated with Sendai virus containing an inactive fusion protein precursor; this virus fails to create target antigens unless the fusion activity is generated by proteolytic cleavage of the precursor (29). Thus, viral antigens must be integrated into the plasma membrane where they are recognized in the context of self H-2.

Our studies with reconstituted membrane vesicles and those of Loh *et al.* (11) with synthetic vesicles containing VSV G-protein demonstrate that primed CTL_p can be triggered by a structural viral protein if the appropriate histocompatibility antigen is present in the same lipid bilayer. Whether viral protein synthesis is required at the induction phase cannot be ascertained by these types of studies. Since induction of anti-VSV responses requires the presence of viral antigen and H-2 antigens in the same lipid bilayer, we suggest that the synthesis and membrane insertion of new viral proteins is a necessary step in the induction event.

The importance of MHC gene products in the recognition of virus-infected cells cannot be overemphasized. In our own studies we have never observed a secondary anti-VSV response with membranes or reconstituted vesicles that failed to elicit a secondary allogeneic response. Furthermore, the anti-VSV response is always restricted to the alloantigens incorporated into the vesicle. Since our cultures contain cytotoxic memory cells that recognize VSV in association with both parental haplotypes ($H\text{-}2^d/2^b$), VSV antigens are not "processed" and reexpressed by an F_1 antigen-presenting cell. It is presumed that MHC restriction reflects the association of viral antigens with serologically detectable H-2 antigens. This view is consistent with the report of Finberg *et al.* (24) who demonstrated that liposomes containing glycoprotein-enriched H-2 antigens and Sendai viral proteins trigger anti-Sendai CTL. Final resolution awaits the preparation of liposomes containing purified H-2 antigens and viral proteins.

Although the nature of the molecular relationship between H-2 molecules and viral antigens is unresolved, current evidence suggests a physical association in the cell surface membrane. For example, T cell-mediated lysis can be blocked with anti-H-2 serum (30) and H-2 specificities are found in purified virions of VSV and Friend virus (Fv) (31, 32). In the case of VSV and Fv infection, this interaction is not random but selective. For example, anti-H-$2K^b$ serum is much more efficient than anti-H-$2D^b$ serum in blocking T cell-mediated lysis of VSV-infected cells and anti-VSV antibody co-caps H-$2K^b$ but not H-$2D^b$ molecules (33). This result correlates with the observation that anti-VSV cytotoxic activity is restricted primarily to the *K*-region in the H-2^b haplotype (33). In contrast, cytotoxic activity against Fv is restricted by the *D*-region of the H-2^b haplotype (30). Similar selective associations between H-$2D^b$ and Fv antigens have been reported (32). Why different viruses selectively associate with K or D-region gene products is an intriguing but unanswered question.

In our studies as well as those of Finberg *et al.* (24), anti-viral effector cells were not induced by vesicles that contained viral antigens but lacked H-2 antigens. We are aware of only one case in which a viral antigen not incorporated into an H-2 antigen containing liposome stimulates T cells. This protein, influenza H, induces H-specific killer cells when cultured *in vitro* with influenza-primed spleen cells (8, 9). Since these experiments were not carried out with F_1 responder cells, it remains unresolved whether H triggers CTL directly or secondarily via a T_H-secreted soluble factor.

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