Vesicular Stomatitis Virus Glycoprotein is Necessary for H-2-Restricted Lysis of Infected Cells by Cytotoxic T Lymphocytes

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Immunology

Vesicular stomatitis virus glycoprotein is necessary for *H-2*-restricted lysis of infected cells by cytotoxic T lymphocytes

(antigen recognition/viral immunity/temperature-sensitive mutants/cell surface antigens)

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ABSTRACT **Vesicular stomatitis virus (VSV) elicited cytotoxic thymus-derived lymphocytes (CTLs) in mice of the BALB/c and three congenic strains (BALB.b, BALB.k, BALB.HTG). CTL lysis of VSV-infected fibroblasts from the four strains was restricted by the target cells' major histocompatibility complex (*H-2*). Target cells were also infected with two temperature-sensitive mutants of VSV, *ts*M and *ts*G in which, respectively, the viral matrix protein and glycoprotein are not expressed at 39° (restrictive temperature) on the infected cell's surface membrane. At the restrictive temperature, cells infected with wild-type VSV or *ts*M were lysed by CTLs, but cells infected with *ts*G were not. The requirement for the glycoprotein on the target cell was also evident from the ability of antisera to the glycoprotein to block completely CTL lysis of VSV-infected cells.**

Lysis of cells with newly acquired foreign surface antigens by cytotoxic thymus-derived (T) lymphocytes (CTLs) probably plays a central role in host resistance to many viral infections and perhaps also in resistance to tumors. The specific attack of CTLs on syngeneic target cells has recently been shown to be governed by the "*H-2* restriction" rule: i.e., to serve as an effective target for CTLs, a cell must have on its surface both the same antigen and the same products of the major histocompatibility complex (called *H-2* in the mouse) as the cells that originally stimulated development of the CTLs (1). Cross-reactions among antigens and among products of the major histocompatibility complex probably account for the occasional instances in which this rule appears to be relaxed (2).

H-2 restriction in the killing of virus-infected target cells may result either from a physical interaction between a major histocompatibility product and a viral product to form a joint antigen or form a requirement for dual recognition by the CTL of both the *H-2* antigen and the viral antigen (1). Whatever the reason, it has thus far proven difficult to inhibit CTL killing with soluble antigens (ref. 3; D. Inbar, A. H. Hale, V. Igras, and H. N. Eisen, unpublished data). Therefore, the competition assays with soluble antigens that have been so useful in studying the antigenic determinants recognized by antibodies have not been fruitful in studying the nature of antigen recognition by CTLs.

To provide an alternative analysis of the antigens recognized by CTLs we have developed a model system in which genetic manipulation of a viral antigen is possible and the antigen is easily purified. We chose vesicular stomatitis virus (VSV) because it specifies a single surface glycoprotein, the G protein, which has been purified and partially characterized (4, 5). Moreover, there are temperature sensitive (ts) mutants available in most of the viral genes (4, 5). The known ts mutants of G protein act after the first glycosylation step and prevent intracellular transport of the protein to the cell surface (6, 7).

We show here that killing of VSV-infected cells by immune syngeneic CTLs is *H-2* restricted. Expression of the G protein on the cell surface is necessary for killing because, at the nonpermissive temperature, cells infected with VSV containing a ts lesion in G protein are not killed by otherwise competent CTLs.

EXPERIMENTAL PROCEDURES

Mice. Female congenic mice of the following strains (*H-2K* and *H-2D* alleles in parentheses) were produced in the Massachusetts Institute of Technology Center for Cancer Research and used at 6–8 weeks of age: BALB/c (*dd*), BALB.b (*bb*), BALB.k (*kk*), and BALB.HTG (*db*).

Cell Culture. Mouse L cells, originally derived from C3H mice (*H-2*^{k} haplotype), and baby hamster kidney (BHK) cells were grown in suspension spinner culture at 37° in minimal Eagle's medium with 7% heat-inactivated fetal calf serum (Gibco). P388D1, a macrophage-like cell line derived from DBA/2 mice (*H-2*^{d} haplotype) (8, 9), was grown in spinner culture at 37° in Dulbecco's modified Eagle's medium. Fibroblastic cell lines were derived as described by Todaro and Green (10) from 17- to 18-day embryos of BALB/c and three congenic strains (BALB.k, BALB.b, and BALB.HTG); these lines were maintained in Dulbecco's modified Eagle's medium with 10% calf serum at 37° (humidified atmosphere, 6% CO_2/94% air). They were passaged 26–36 times before use.

Virus. Stocks were prepared by infecting baby hamster kidney cells (4×10^5 cells per ml) with twice-plaque-purified VSV Indiana (11, 12) at a multiplicity of 0.1. Virus was purified away from defective particles and concentrated by sucrose gradient centrifugation and sedimentation (13). The ts mutants were *ts*M301 (III), called here *ts*M because it is defective in VSV matrix (M) protein, and *ts*M501 (V), called here *ts*G because it is defective in G protein (6, 7).

Immunization. Mice were injected intraperitoneally or intravenously with various amounts of wild-type virus inactivated in 20% sucrose in phosphate-buffered saline (8.0 g of NaCl, 0.2 g of KCl, 1.8 g of $Na_2HPO_4 \cdot 7H_2O$, and 0.2 g of KH_2PO_4 in 1 liter, pH 7.4) with ultraviolet light to a final titer of 10^2–10^3 plaque-forming units (PFU).

Preparation of Effector Cells. Spleen and peritoneal exudate cells (PECs) were used as effectors in cytotoxicity assays. These cells were harvested and freed of erythrocytes as described (14).

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Abbreviations: CTLs, cytotoxic T lymphocytes; VSV, vesicular stomatitis virus; G, VSV glycoprotein; ts, temperature sensitive; M, VSV matrix protein; PFU, plaque-forming units determined on L cells; PEC, peritoneal exudate cell

Preparation of Targets. P388D1 was plated at 0.5–1 × 10^4 cells per well and 3T3 fibroblasts were plated at 1–2 × 10^3 cells per well in tissue culture plates (6-mm diameter wells, Linbro Scientific, Inc., Hamden, CT) and incubated for 24–36 hr before infection with VSV (multiplicity of infection, 10–25). Four hours later, the cells were labeled by incubating them for 1.5 hr with ^{51}Cr ($Na_2{}^{51}CrO_4$ at 1 mCi/ml, 25 μl per well, New England Nuclear); then they were washed, and effector cells were added.

Target Cell Lysis of CTLs and Lysis-Inhibition. Effector cells (serially diluted PECs or spleen cells) were usually incubated with target cells at 37° (final volume, 200 μl in 6-mm wells of Linbro plates). However, when ts mutants were used, the target cells were infected, labeled with ^{51}Cr, and assayed for lysis at 34° and 39° (permissive and nonpermissive temperatures, respectively). After 4 hr, each well was individually agitated with a pasteur pipet, the entire plate was centrifuged (450 × *g*, 5 min), and supernatant radioactivity (representing ^{51}Cr released by lysed cells) was measured in a Packard Auto-Gamma scintillation spectrometer. The sedimented cells were dried, dissolved in 200 μl of 1 M NaOH, and also assayed for radioactivity. Specific lysis was defined as 100 (*IR* − *NR*/*T* − *NR*), in which *IR* and *NR* represent percent ^{51}Cr release by target cells incubated with immune and normal lymphocytes, respectively, and *T* is the total target cell radioactivity (100%).

To test the ability of antibodies to block CTL activity, target cells were incubated (30 min, 37°) with serial dilutions of various antisera, and effector cells (plus additional antiserum to maintain the antiserum dilution) were then added at effector-to-target ratios that caused 15%–30% lysis of the targets. Cell lysis was then assayed as described above.

Antiserum to G Protein. The envelope glycoprotein of VSV (G protein) was extracted from sucrose-gradient purified virus with Nonidet P40 (15). To remove contaminants, G protein preparations were passed through Sephadex G-75 in phosphate-buffered saline/0.2% deoxycholate. Electrophoretic analysis showed that the void volume contained G protein as monomers and dimers; it also contained trace amounts of some cellular proteins, but the other viral proteins were not detectable.

Rabbits were injected once with 50 μg of purified G protein in complete Freund's adjuvant and twice (3- to 4-wk intervals) with 25 μg of G protein in incomplete adjuvant. Anti-G antibodies became evident only after additional injections, at 1-month intervals, of 50 μg of glutaraldehyde-crosslinked G protein in incomplete adjuvant. The antisera at a 1:100 dilution decreased infectivity of the test virus by four to five orders of magnitude. Specificity was established by polyacrylamide gel electrophoresis (in 0.1% sodium dodecyl sulfate) of immune precipitates (16) prepared with anti-G serum and lysates of VSV-infected cells that had been grown in [^{35}S]methionine (16): about 95% of the specifically precipitated [^{35}S]protein migrated with G protein.

Other Antisera. Antiserum to disrupted VSV virions was generously provided by Alice Huang, Harvard Medical School. One sample of anti-thy 1.2 sera (AKR mice immunized with C3H thymocytes) was a generous gift from Michael Bevan, Massachusetts Institute of Technology; another sample was obtained from Litton Laboratories (Bethesda, MD).

Media. For all cell-mediated cytotoxicity assays, the cells were grown, washed, and incubated in RPMI 1640 (Gibco) supplemented with fetal calf serum, amino acids, sodium pyruvate, penicillin/streptomycin, and 2-mercaptoethanol as described (14). For assays with antibody and complement, sera were diluted and cells were washed with RPMI 1640/0.1% gelatin.

RESULTS

Eliciting CTLs *In Vivo*. Cytotoxic cells that were active over a wide range of effector/target ratios were more consistently elicited in mice injected with UV-inactivated VSV than in those injected with infectious virus, perhaps because proliferating T cells are unusually vulnerable to VSV infection (17–19).

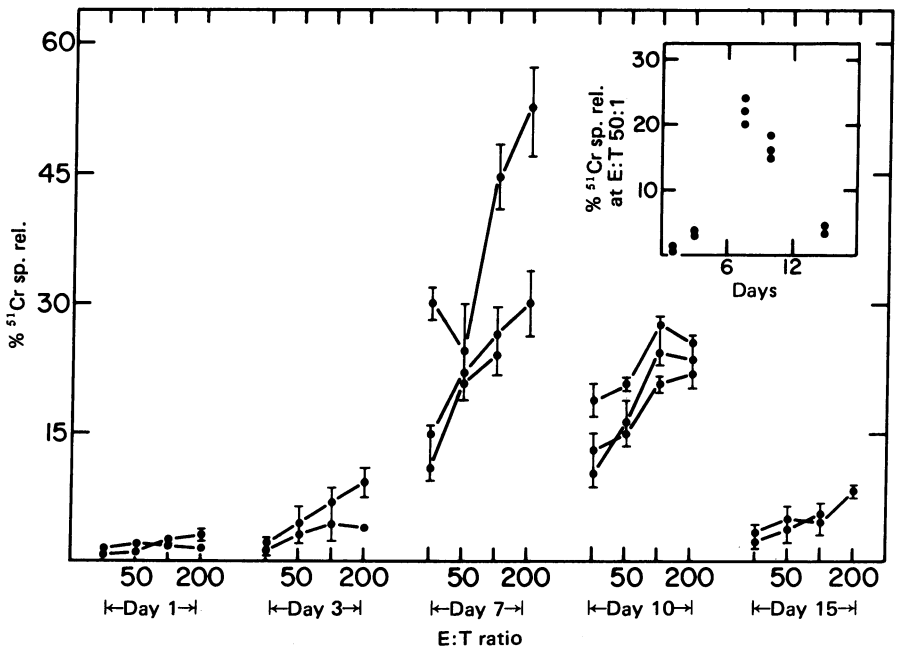

FIG. 1. Time course of development of cytotoxic T lymphocytes. Spleen cells removed at various times after one intravenous injection of BALB/c mice with inactivated VSV (2 × 10^8 PFU before and 10^2–10^3 after irradiation) were tested for ability to lyse infected P388D1 cells. E:T, ratio of numbers of effector (spleen) to target cells in the cytotoxicity assay. (*Inset*) Summary of results for individual mice at E:T = 50:1.

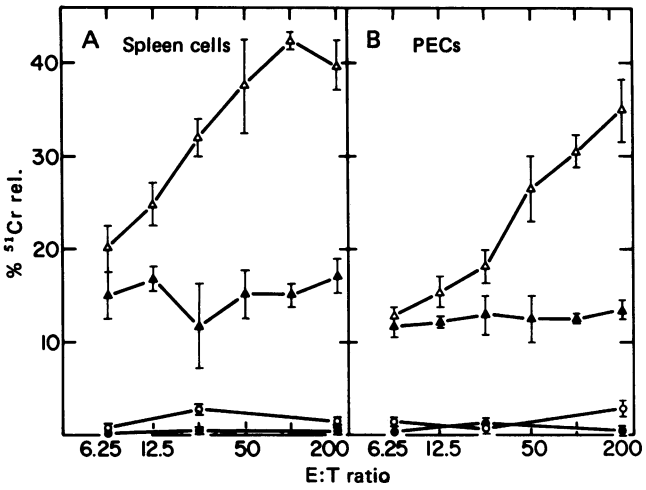

FIG. 2. Lysis of VSV-infected and uninfected P388D1 cells by spleen cells (*A*) and PECs (*B*) from normal and VSV-immunized mice at various effector/target (E:T) ratios. △, Infected P388D1 cells + immune spleen cells or PECs; ▲, infected P388D1 cells + normal spleen cells or PECs; ○, uninfected P388D1 cells + immune spleen cells or PECs; ●, uninfected P388D1 cells + normal spleen cells or PECs.

Preliminary studies indicated that, with UV-inactivated virus, cytotoxic cells could be elicited by a single intravenous or intraperitoneal injection. Subsequent immunizations were carried out with one injection of 2×10^8 PFU inactivated to 10^2–10^3 PFU. Spleen cells and PECs were harvested 6–8 days later, based on the time course shown in Fig. 1. After intravenous injection of virus, cytotoxic cells were regularly present in spleen (Fig. 2*A*) but only rarely in PECs (data not shown); after intraperitoneal injection they were regularly present in PECs (Fig. 2*B*) but only occasionally in spleen (data not shown). Target cells were not lysed by spleen cells or PECs from nonimmunized mice. The cytotoxic cells were T cells (CTLs) because all cytotoxic activity of spleen cells and PECs from VSV-immunized mice was specifically eliminated by treating the cells with anti-thy 1.2 serum (AKR antiserum to C3H thymocytes) plus complement (data not shown).

***H-2* Restriction of CTLs.** As shown in Table 1, at an effector/target cell ratio of 50:1, CTLs from VSV-immunized BALB/c mice and the three congenic strains caused significant lysis only when the target cells shared at least one *H-2D* or *H-2K* allele with the stimulator and effector cells. For example, CTLs from immunized BALB.b (*bb*) mice caused specific lysis of infected 3T3 fibroblastic targets derived from the BALB.b and BALB.HTG (*db*) strains but not for the corresponding BALB/c (*dd*) or BALB.k (*kk*) target cells. Although Table 1 shows virtually no killing over the background level across *H-2* differences, when the effector/target ratio was increased to 200:1, up to 20% of the lysis observed with the homologous target was found with *H-2* different targets.

Infection of Target Cells with ts Mutants. There are two known VSV-specified proteins associated with the surface of infected cells: G protein found on the exterior surface of the plasma membrane and M protein found mainly on the interior surface. To investigate whether one or both of these proteins are parts of the targets for CTLs, ts mutants of VSV were used. At the nonpermissive temperature (39°), *ts*M makes a defective M protein that is rapidly degraded after its synthesis and *ts*G makes a defective G protein that does not move to the cell surface from its site of synthesis on intracellular membranes (6, 7).

Cells infected with *ts*M were specifically lysed at both restrictive and permissive temperature by CTLs from mice immunized with wild-type virus, and they were lysed to virtually the same extent as targets infected with wild-type virus (Fig. 3 *A* and *B*). In contrast, cells infected at the nonpermissive temperature (39°) with *ts*G failed to serve as targets whereas, at the same temperature, cells infected with wild-type virus were readily lysed (Fig. 3 *D*). At 34°, cells infected with *ts*G express the G protein normally (6, 7) and were also readily lysed (Fig. 3 *C*). In corroboration of these findings, lysis of target cells infected with wild-type VSV was completely blocked by antisera to G protein as well as by antiserum to virions (Fig. 4).

DISCUSSION

The present study shows that lysis of VSV-infected cells by CTLs from VSV-immunized mice is subject to *H-2* restriction. Similar restriction has already been well established in the immune responses to infection by several other viruses (21–25). The limited number of proteins specified by the VSV genome (4–5) and the existence of mutants with biochemically defined

Table 1. *H-2* restriction in lysis of 3T3 fibroblasts from congenic BALB mice

Spleen cells from*		Targets†				
Strain	Status	P388D1 (*dd*)	3T3 BALB/c (*dd*)	3T3 BALB.b (*bb*)	3T3 BALB.k (*kk*)	3T3 BALB.HTG (*db*)
BALB/c	N	10.7 ± 2.8	17.7 ± 2.1	22.7 ± 1.1	21.7 ± 2.7	27.8 ± 1.9
	I	**41.7 ± 5.8**	**32.1 ± 6.2**	25.8 ± 3.5	25.7 ± 4.2	**38.1 ± 2.8**
BALB.b	N	12.2 ± 3.7	16.3 ± 1.7	17.5 ± 3.1	15.6 ± 3.2	17.7 ± 2.8
	I	15.1 ± 4.8	17.5 ± 2.4	**32.7 ± 3.3**	14.8 ± 1.8	**29.8 ± 3.7**
BALB.k	N	14.8 ± 3.1	20.7 ± 2.9	18.7 ± 2.4	21.0 ± 2.7	17.0 ± 2.0
	I	16.1 ± 3.7	22.8 ± 3.1	23.8 ± 1.7	**36.8 ± 3.7**	22.0 ± 1.7
BALB.HTG	N	13.9 ± 2.8	19.7 ± 3.1	21.0 ± 1.7	17.2 ± 1.8	18.1 ± 1.9
	I	**35.8 ± 5.1**	**41.7 ± 5.8**	**37.1 ± 3.8**	21.1 ± 2.7	**39.1 ± 6.1**

* Mice were immunized by one intravenous injection of UV-irradiated virus. Immune effectors (I) were spleen cells taken 7 days after the injection; controls were normal spleen cells (N) from uninjected mice of the same strain.

† The targets, labeled with ^{51}Cr and infected with VSV (multiplicity of infection, 25), were P388D1 cells or 3T3 fibroblasts from the indicated strains (*H-2K* and *H-2D* alleles in parentheses), all tested at an effector/target cell radio of 50:1. Values are %^{51}Cr released, shown as mean ± half the range of duplicates. Mean values ≥ 30% were observed only under conditions in which requirements for *H-2* restriction were met (**boldface**).

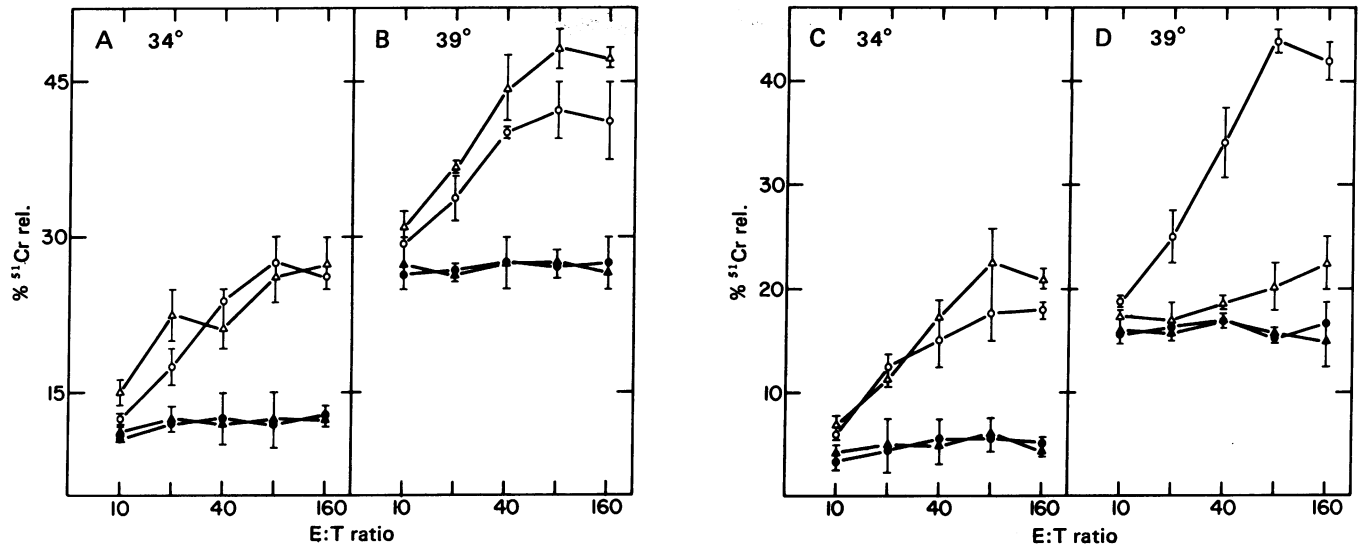

FIG. 3. Lysis of P388D1 target cells infected with wild-type VSV, *ts*M (*A* and *B*), or *ts*G (*C* and *D*). Infected P388D1 target cells (multiplicity of infection, 10–25) were tested at 34° (*A* and *C*) and 39° (*B* and *D*) (permissive and nonpermissive temperatures, respectively) for susceptibility to lysis by spleen cells from mice injected intravenously with wild-type VSV or from uninjected (control) mice. △, Mutant-infected targets + immune spleen cells; ○, wild-type VSV-infected targets + immune spleen cells; ▲, mutant-infected targets + normal spleen cells; ●, wild-type VSV-infected targets + normal spleen cells. Slight lysis at high levels of effector cells in *D* could mean that a small proportion of the CTLs are directed to an antigen other than G protein or, more likely, that a small amount of G protein is present on cells infected with *ts*G at the nonpermissive temperature (20).

defects (e.g., refs. 4–7) make the VSV system a particularly promising one for identifying unambiguously the viral antigen required for CTL activity.

Cells infected by *ts*M were equally susceptible to lysis by CTLs at permissive (34°) and nonpermissive (39°) temperatures, whereas cells infected with *ts*G were lysed only at the permissive temperature (34°), at which they express G protein on the cell surface. The necessity for cell surface G protein is also suggested by the ability of antisera to G protein to block lysis of infected cells. Recent studies of CTL lysis of cells infected with recombinant and variant influenza viruses have also pointed to particular viral proteins (hemagglutin, matrix protein) as being necessary for lysis by *H-2* restricted CTLs (22, 23).

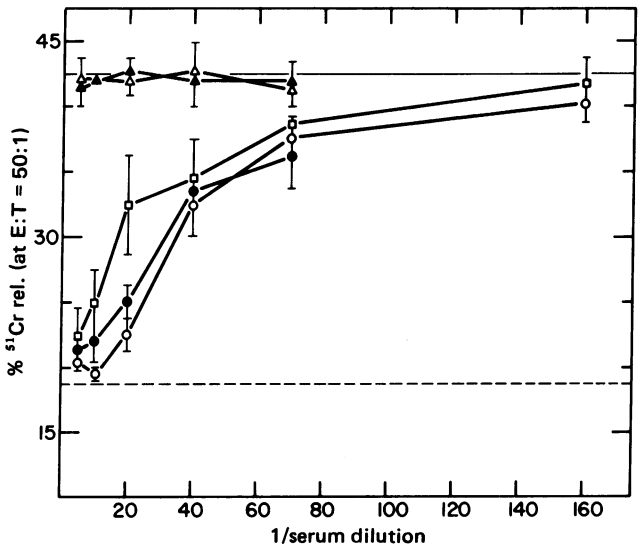

FIG. 4. Rabbit antisera to disrupted virions (anti-VSV) or to purified G protein (anti-G) inhibited lysis of VSV-infected P388D1 target cells by CTLs from spleens of BALB/c mice injected intravenously with irradiated wild-type virus (E:T ratio, 50:1)). In one experiment, anti-G was adsorbed with 2×10^8 cells of VSV-infected mouse L cells before being tested as an inhibitor. Adsorbed and unadsorbed sera at dilutions of 1:5 to 1:160 were incubated with the target cells before addition of effector CTLs. Uninfected L cells did not adsorb the anti-G antibodies (data not shown). Solid horizontal line (at 42%–45% ^{51}Cr release) represents uninhibited lysis by CTLs. Broken horizontal line (at 17.5%) represents ^{51}Cr release in presence of spleen cells from nonimmunized mice. ●, Anti-VSV sera; □, anti-G serum (from rabbit 1); ○, anti-G serum (from rabbit 2); ▲, anti-G (from rabbit 2) after adsorption with VSV-infected L cells; △, normal rabbit serum.

The present results are particularly significant in relation to the general problem of identifying the cell surface antigens that are recognized by CTLs. Genetic and serological analyses currently provide the only approaches available for identifying these antigens. But neither of these approaches leads to unequivocal results. Thus, correlations between a target cell's genome and its susceptibility to lysis only establish linkage, not identity, between a particular allelic product and key cell surface antigens. Under some circumstances, as with the ts VSV mutants and influenza virus recombinants, it is possible to show that a particular cell surface molecule is essential for lysis, but not that the molecule is itself recognized by CTLs. Likewise, antibody blockade of lysis may be due to steric hindrance by bound antibody molecules rather than to competitive binding of the same antigenic determinants by both the blocking antibodies and CTLs (26, 27).

There is also some uncertainty about the products of the *H-2* complex that restrict antigen recognition by syngeneic CTLs. In a recent study of a plasmacytoma, for instance, tumor cells that reacted perfectly well with anti-*H-2* alloantibodies lost their reactivity with allogeneic anti-*H-2* CTLs (14) and with *H-2* syngeneic CTLs that were directed to other antigens on the tumor cells (A. H. Hale, J. H. Russell, and H. N. Eisen, unpublished data). Studies of mutations at the *H-2K* and *H-2D* loci of C57BL/6 and BALB/c mice also raise the possibility that the cell surface molecules recognized by CTLs may not be the glycoproteins recognized by anti-*H-2* antibodies (27–29). In view of all these uncertainties there is an almost complete void

in our understanding of the molecular properties of the cell surface antigens recognized by CTLs on syngeneic tumor cells and on virus-infected cells.

Although genetic variants of target cells and antibody blockade of lysis can provide valuable leads, unequivocal identification of the antigens recognized by CTLs requires more direct evidence, such as the demonstration that a purified substance binds specifically to CTLs or elicits the development of these T cells or can be used to construct particles that are active in either capacity. Because the G protein of VSV can be readily purified in relatively large amounts, it may prove to be particularly helpful for developing general means for identifying the antigens recognized by CTLs, including those antigens for which genetic variants and antisera are not available.

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