The Soluble Viral Glycoprotein of Vesicular Stomatitis Virus Efficiently Sensitizes Target Cells for Lysis by CD4+ T Lymphocytes

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The soluble glycoprotein Gs of vesicular stomatitis virus (VSV), at approximately 10^4 molecules per cell, sensitized target cells for lysis by clones of CD4+ cytolytic T lymphocytes (CTL). In addition to lysis, the clones responded by proliferation and interleukin-2 release. Targets sensitized by Gs competed effectively with VSV-infected cells for recognition. Immune cytolysis by these CD4+ CTLs was restricted by class II major histocompatibility complex (MHC) antigens and was specific to VSV. The specific class II MHC antigen which was restricting for each clone remained the same whether the targets were sensitized by infection with VSV or by exogenously added soluble antigen. Sensitization by Gs appeared to require prior processing because the antigen-presenting cells that were fixed prior to exposure to Gs failed to be recognized by the CTL clones. The high efficiency of this uptake and processing was suggested by the inability of Gs at concentrations up to 10^7 per cell to block superinfection by VSV or to effect the RNA-synthetic machinery of uninfected cells. Also, Gs failed to hemolyze sheep erythrocytes when there was hemolysis by virions or an amino-terminal peptide of the VSV glycoprotein. Extrapolation of these results to viral diseases was possible because soluble viral glycoproteins were naturally synthesized during many viral infections and class II MHC antigens were inducible in cells of nonlymphoid origin. Therefore, CD4+ CTLs may be important participants in increasing virus-induced pathology, especially among adjacent uninfected cells.

Infectious agents such as viruses have a complex relationship with the immune system of their hosts. We have studied the in vitro interactions between cloned murine T lymphocytes and defined antigens of vesicular stomatitis virus (VSV). T-cell clones expressing the CD3+, CD4+, CD8- phenotype, specific for the glycoprotein antigen of VSV, have been derived from BALB/c-H-2^{dm2} (dm2) mice (M. J. Browning, A. S. Huang, and C. S. Reiss, *J. Immunol.*, in press). These cells are restricted by class II major histocompatibility (MHC) antigens and recognize and lyse VSV-infected Ia+ cells. These cytolytic T lymphocytes (CTLs) also lyse cells treated with UV-irradiated virus, nonreplicating defective interfering particles, and micelles containing only the viral glycoprotein G (Browning et al., in press). They differ from classic CTLs with a CD8+ phenotype, which are restricted by class I MHC antigens and lyse only infected cells endogenously producing viral proteins. Similar target specificities among CD8+ or CD4+ CTLs have been reported for influenza virus (21).

During studies to define these CD4+ CTL clones further in relation to antigen processing and epitope mapping, it was found that a natural fragment of the VSV glycoprotein, called soluble glycoprotein Gs (13, 18), sensitized antigen-presenting cells (APCs) as targets for the CD4+ CTL clones. This fragment, which represents almost all but 22 amino acids of the ectodomain of G, accumulates in the extracellular medium of infected cells (4, 17). It is not a degradation product of virions or infected cells and can be produced even when infected cells are nonpermissive for viral replication (17). It is also shed from persistently infected cells. For wild-type strains of VSV, one in every six G molecules is

cleaved in the rough endoplasmic reticulum shortly after synthesis, transported to the cell surface, and shed into the medium (5, 8, 18). *tsO45*, a mutant of VSV, codes for a defective glycoprotein at the nonpermissive temperature, so that almost all of the glycoprotein is cleaved and found in the medium together with spikeless virions containing just the membrane-anchoring fragment (6, 20).

Compared with virions or glycoprotein-containing micelles, Gs was found to be extremely efficient in terms of the minimal number of molecules required to sensitize class II MHC-expressing target APCs. Because this was a surprising result and may have important implications for immunopathogenesis, further studies were carried out to define the parameters of this cell killing and to ensure that the reaction was specific in relation to the sensitizing antigen, the MHC restriction, and the reactivity of the CD4+ CTL clones. This paper presents these results and discusses the immunopathological potential of soluble viral glycoproteins.

MATERIALS AND METHODS

Viruses, cells, and reagents. VSV of the Indiana serotype (San Juan strain), other viruses used as controls, and all of the cell lines used in this study have been described previously (Browning et al., in press). In some of the figures, infectious VSV of the Indiana serotype has been designated DC-2B, indicating that sucrose-gradient-purified virus was used. The soluble glycoprotein Gs was isolated from the medium of infected cells by affinity chromatography, using a polyclonal goat antiserum to VSV (5). Recombinant gp120 (MicroGeneSys, Inc.) was a kind gift from S. Herrmann.

Methods of procedure. The derivation and characterization of dm2 anti-VSV CTL clones have been described in detail previously (Browning et al., in press). Assays of lymphocyte

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FIG. 1. Low concentrations of Gs sensitize target cells for cytotoxicity by VSV-specific, class II MHC-restricted T-lymphocyte clones. ^{51}Cr -labeled A20 cells were incubated with Gs at various concentrations or infected with VSV (DC-2B) at a multiplicity of infection of 10 for 2 h and then washed three times prior to use as target cells. They were incubated for 4 h together with four different T-cell clones, all characterized previously to be class II MHC-restricted, anti-VSV, G-protein-specific CTL clones (Browning et al., in press). Several effector/target cell ratios were determined, but only one, 3:1, is shown. Symbols: ■, uninfected; ▨, DC-2B; ▩, Gs (3 µg/ml); ▪, Gs (0.3 µg/ml); □, Gs (0.03 µg/ml) B.

function (CTL activity, proliferation, and cytokine release) were carried out as described by Reiss and her colleagues (24; Browning et al., in press). Gs sensitization of target cells for CTL assays involved incubation of the cells with Gs (or control antigens) for 2 h at 37°C followed by extensive washing prior to their use as targets in CTL assays.

RESULTS

Titration of the amount of Gs needed to sensitize target cells. Previous experiments had shown that G at 6 µg/ml readily sensitized A20 target cells for immune cytotoxicity by CD4+ CTL clones. Using a similar protocol, we investigated the ability of Gs to sensitize target cells for these CTL clones and to determine the lower limits of Gs needed for sensitization. Several individual CTL clones were tested against ^{51}Cr -labeled A20 target cells treated with different concentrations of Gs or with infectious VSV. Gs at 30 ng/ml, representing 3×10^4 molecules per cell, gave a positive response (Fig. 1). This was in contrast to previous experiments with G-containing micelles or UV-irradiated VSV in which at least 100 times more G was needed to obtain the same degree of recognition and lysis (Browning et al., in press). Optimal sensitization was obtained with Gs at 3 µg/ml with one of the clones, G11. At this concentration, Gs sensitized targets for cytotoxicity with an efficiency equal to infectious VSV (Fig. 1).

Specificity of CTL clones for Gs. These CTL clones recognize only VSV-infected cells and not influenza virus-infected cells (Browning et al., in press). Moreover, they are serotype

FIG. 2. Specificity of the viral glycoprotein in target cell sensitization for class I-restricted CTL clones. Labeled A20 cells were sensitized with VSV (DC-2B), with Gs, with gp120, or with nothing for 2 h, washed, and then incubated with the same four CTL clones shown in Fig. 1 at an effector/target cell ratio of 4:1. Symbols: □, uninfected A20 cells; ▨, VSV infected; ▩, Gs (1 µg/ml); ▪, Gp120 (2 µg/ml).

specific, recognizing only the Indiana serotype of VSV and not its New Jersey counterpart. To ensure that this recognition was specific for Gs and was not mediated by unrelated viral glycoproteins, targets were sensitized with gp120, the external glycoprotein of the human immunodeficiency virus (25). Like Gs, this antigen is a soluble viral glycoprotein and, therefore, represents an appropriate specificity control. Gp120 failed to sensitize target cells for recognition by these CTL clones, whereas control cells, sensitized by infectious VSV or Gs, resulted in a 40% or better release of ^{51}Cr in this experiment (Fig. 2). Also, Gs at 1 µg/ml was as effective or nearly as effective as infectious VSV for sensitizing target cells for all four T-lymphocyte clones shown. Therefore, gp120 failed to sensitize, suggesting that Gs was the specific antigen recognized by these clones.

Gs-sensitized targets competed with VSV-infected targets. The previous experiments indicated that uninfected Ia+ cells could be sensitized with Gs for immune cytotoxicity by VSV-specific CD4+ CTLs. To determine whether Gs-sensitized targets competed with VSV-infected targets for recognition by these CTLs, "cold target" inhibition experiments were performed. ^{51}Cr -labeled, VSV-infected A20 (Ia+) target cells were exposed to the CTL clones in the presence of increasing numbers of unlabeled cold target cells not sensitized with antigen, sensitized by DC-2B VSV infection, or sensitized by Gs at 1 µg/ml. Figure 3 shows the results with one representative CTL clone, 27H1. Inhibition of cytotoxicity was seen when either VSV-infected or Gs-sensitized cold A20 target cells, expressing class II MHC antigens, were added. However, with sensitized cold targets (P815 cells) that did not express the class II MHC-restricting element, competition was not detected. These results demonstrate that uninfected Ia+ cells, exposed to Gs, competed effectively with VSV-infected cells for cytolytic recognition by the CTL clones. Moreover, the failure of Gs-sensitized or VSV-infected P815 cells to compete confirm that class I MHC antigens are not recognized by these CTL clones.

FIG. 3. Cold target inhibition assays with Ia+ and Ia- targets sensitized by Gs. A20 cells (left panel) and P815 cells (right panel) were sensitized with Gs, infectious VSV (DC-2B), or nothing and used at various concentrations to compete with a constant number of ^{51}Cr -labeled A20 cells infected with VSV as targets for lysis by clone 27H1 at an effector/hot target cell ratio of 3:1. Symbols: $\square$, uninfected; $\blacklozenge$, DC-2B; $\square$, Gs (1 $\mu\text{g}/\text{ml}$).

Gs-sensitized cells induced proliferation and release of IL-2 by the CTL clones. To determine whether the CTL clones responded to Gs by other functional assays of T-lymphocyte activation, proliferation and interleukin-2 (IL-2) release were measured under conditions identical to those used for eliciting the CTL response. Table 1 shows representative results obtained with clone 9-F1. Gs induced proliferation of the CTLs and their release of IL-2. At 1 $\mu\text{g}/\text{ml}$, however, Gs appeared to be less effective at these inductions than at sensitizing targets for immune cytotoxicity. Nonetheless, Gs-presenting cells were able to induce the usual "helper" phenotype of these CD4+ T-lymphocyte clones.

Further mapping of class II MHC restriction. To determine

TABLE 1. Gs-pulsed APCs induce proliferation and IL-2 release as well as cytolytic activity^a

APC	Proliferation (cpm)	IL-2 release (U/ml)	CTL activity (% lysis)
A20, uninfected	721	1	0
A20, VSV MOI of 10	60,322	752	32
A20, Gs at 1 $\mu\text{g}/\text{ml}$	10,611	76	32

^a Proliferation of clone 9-F1 was measured by incorporation of [^3H] thymidine into acid-precipitable radioactivity during the last 16 h of a 3-day incubation as described by Browning et al. (in press). Production of IL-2 by the clone was detected by the factor-dependent cell line CTLL20, as described previously (Browning et al., in press). CTL activity was determined by ^{51}Cr release assays with A20 target cells as described in the legend to Fig. 1. MOI, Multiplicity of infection.

which class II antigen was restrictive for the recognition of Gs, L cells transfected and expressing either the I-E^d or the I-A^d antigen on their surface (3) were used as APCs with either Gs at increasing concentrations or infectious VSV, as represented by *tsG41*, at a multiplicity of infection of 1. The proliferative response of the CTL clone was measured by their incorporation of radioactive thymidine. Figure 4 shows the results with a representative clone, 9-F1, whose recognition of Gs was restricted only by the I-E^d antigen, the MHC product which also restricted recognition of *tsG41*-infected cells by the same clone. Sensitized L cells transfected by the other class II antigen (I-A^d), as well as those transfected by only the *tk* vector, were not recognized. Dilution of Gs to as little as 10 ng/ml resulted in a detectable response. The specificity of the restriction remained consistent whether the APCs were infected with VSV or treated with Gs.

APCs processed Gs for CTL recognition. To test whether Gs was bound to the outside of APCs or had to be processed prior to recognition by the CTL clones, groups of APCs were first fixed in paraformaldehyde and then treated with Gs, pulsed with Gs and then fixed, or exposed to Gs and not fixed. The APCs were then cultured with clone 9-F1, and the proliferative response of the clone was estimated by radioactive thymidine incorporation. Figure 5 shows that prior fixation drastically reduced the ability of the cells to be recognized by the 9-F1 clone, whereas cells that were first sensitized and then fixed stimulated the clone to proliferate,

FIG. 4. Proliferative response of clone 9-F1 to Gs is restricted by H-2I-E^d. Mouse L929 cells, transfected with H-2I-A^d, H-2I-E^d, or vector alone, were irradiated and cultured with clone 9-F1 in the presence of increasing concentrations of Gs. VSV (tsG41; multiplicity of infection, 1)-infected cells were used as controls for infectious virus. Triplicate cultures of APCs were mixed with 2×10^4 CTLs per well. Proliferation of the T cells were measured by their incorporation of radioactive thymidine during the last 18 h of a 64-h culture period. Symbols: $\blacklozenge$, I-E^d; $\blacksquare$, I-A^d; $\circ$, TK⁺.

although at only 3% when compared with unfixed cells. These results indicate that Gs was processed by APCs before its epitopes could be recognized by the class II MHC-restricted, VSV-specific CTL clones.

Gs failed to block VSV infection and had no cytotoxic or

FIG. 5. Processing by APCs is required for T-cell recognition of Gs. Splenocytes from dm2 mice were treated with Tris-NH₄Cl and 2,000 rads and then fixed with paraformaldehyde (0.25%) and then exposed to Gs (1 μ g/ml for 6 h), fixed after exposure to Gs, or exposed to Gs only. These splenocytes at 10^5 per well, in triplicate, were then mixed with cloned 9-F1 cells (2×10^4) and incubated for 48 h. [³H]thymidine at 1 μ Ci/well was added, and incubation was continued for an additional 16 h prior to determination of the amount of radioactivity incorporated by liquid scintillation spectroscopy. Symbols: $\square$ (cross-hatch), untreated APCs plus clone 9-F1; $\square$ (diagonal lines), APCs plus Gs (1 μ g/ml) plus 9-F1.

hemolytic activity. It had been assumed previously that, since Gs contained most of the ectodomain of G, an excess of Gs would block receptors on cells and prevent infection by VSV. However, if Gs is rapidly taken up by cells and processed, it may not be able to block at all. To test whether Gs had any blocking abilities, a sensitive assay, based on [¹⁴C]uridine incorporation by VSV-infected cells in the presence of dactinomycin, was used to determine VSV entry and growth in cells treated with Gs at concentrations of 1×10^6 to 6×10^7 molecules per cell. No inhibition of viral RNA synthesis could be detected when Gs was present (Fig. 6A). Even in the presence of a great excess of Gs to potential cellular receptors, VSV was able to attach and enter cells. Similar observations were made for the rabies virus soluble glycoprotein (7). These results suggest that Gs did not stably associate with cells at their surface, but may have been rapidly taken up and presumably processed into smaller fragments.

To determine whether the uptake of Gs had any direct effect on the target cells, its cytotoxic and hemolytic potential was examined. Cells were exposed to Gs at 2.7×10^7 molecules per cell, and their ability to synthesize RNA was measured at three different pHs. Although acid pH was inhibitory by itself, Gs had no noticeable effect on the RNA-synthetic machinery of cells over a 3-h period (Fig. 6B).

Since Gs contains the amino terminus of G, which has been shown to be apparently responsible for the hemolysis activity of VSV (28), Gs was tested under conditions which resulted in the lysis of sheep erythrocytes by virions or by the six-amino-acid peptide, representing the amino terminus of G. Table 2 shows that Gs was unable to cause any hemolysis over the background levels. Therefore, Gs must differ in some significant way from the ectodomain of virion-associated G under these conditions.

DISCUSSION

The naturally synthesized soluble glycoprotein Gs of VSV very efficiently sensitized uninfected, Ia^d-expressing APCs. These cells were recognized by specific CD4⁺ T-lymphocyte clones restricted by class II MHC antigens. The clones responded by lysis of the targets as well as by clonal proliferation and release of IL-2. These conclusions were arrived at by studying 10 independent isolates of T-lymphocyte clones obtained from the dm2 mouse after in vivo priming with VSV. Previous studies with the same clones showed that they were specific for the glycoprotein of VSV, either endogenously synthesized by infected cells or exogenously added to class II MHC-expressing APCs (Browning et al., in press).

The lysis of Gs-sensitized APCs differed from the bystander effect, a term used to describe the nonspecific killing of "innocent" neighboring cells by toxins released from stimulated macrophages or other cells (9, 12). It also differed from lysis resulting from antibody-antigen complexes because neither antibody nor complement appeared to be required. Because Gs-sensitized targets competed successfully with virus-infected cells for recognition, this mechanism of cell killing may be an important cause of the observed immunopathology during viral infections.

The minimal amount of Gs needed to sensitize target cells in vitro was on the order of 3×10^4 molecules per cell. This is in contrast to other forms of the glycoprotein antigen, 100 times or more of which are needed (Browning et al., in press). Why Gs was so efficient in sensitizing target cells for recognition by CD4⁺ lymphocytes is unknown. The hemo-

FIG. 6. Viral RNA or host RNA synthesis in the presence of Gs. Chinese hamster ovary cells at 4×10^6 were incubated in 2 ml of medium and mixed with Gs at the indicated concentrations. (A) VSV was added at a multiplicity of 20 together with 10 μg of dactinomycin. After incubation at 37°C for 30 min, [^{14}C]uridine (0.1 μCi) was added and trichloroacetic acid-precipitable radioactivity was determined hourly. (B) Only the cells mixed with 10 μg of Gs were compared with untreated cells at three different pHs, buffered by the organic buffers HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]. After incubation at 30 min at 37°C , radioactive uridine was added and the cellular RNA synthesis was assayed as for panel A.

lysis results suggest conformational differences between Gs and G. Yet examination of trimer formation by centrifugation in sucrose at low pH showed that Gs trimerized as well as G (B. A. Onile, S. L. Chen, W. Keil, and A. S. Huang, unpublished observations). Other studies indicate that Gs is hydrophobic by measuring their charge shift in ionic detergents (18) and by phase partitioning in aqueous Triton X-114 (S. L. Chen and A. S. Huang, unpublished observations). These properties and its instability at the cell surface may account for the efficiency of Gs in sensitization of target cells.

Siliciano et al. (30), using the soluble gp120 of human immunodeficiency virus, found a similar efficiency compared with virions or peptides. In this case, T cells and clones from normal human donors with the CD4⁺ phenotype were expanded in the presence of human immunodeficiency virus

antigens. The clones recognize and lyse target cells sensitized by gp120 in the context of class II MHC (Ia) antigens. Low concentrations of gp120, in the order of 10^4 molecules, are effective in targeting cells for cytolysis. Because activated human CD4⁺ T cells, in contrast to mice, express class II MHC antigens, the authors postulated that gp120 shed in vivo may account for the depletion of uninfected CD4⁺ T cells in the pathophysiology of acquired immunodeficiency syndrome. Such possibilities have become more likely since the finding of a specific CD4⁺ T-lymphocyte response in patients with acquired immunodeficiency syndrome (29).

Studies on VSV and human immunodeficiency virus soluble glycoproteins may be extrapolated to other viruses. The lysis of uninfected cells exposed to soluble viral glycoprotein by class II MHC-restricted CTLs and their competition for recognition with virus-infected cells may play an important role in the immunopathology of viral infections. CTLs restricted by class II products have been found in response to several human viral infections in addition to acquired immunodeficiency syndrome, such as influenza, herpes-, and measles viruses (11, 15, 31). Also, several enveloped viruses are known to produce cleaved glycoprotein fragments during infection of cells. In addition to VSV, they are rabies virus (7), herpesvirus (14), retroviruses (1), and respiratory syncytial virus (10). This shedding is probably a common feature of viruses encoding membrane-spanning glycoproteins. Therefore, this phenomenon may be relevant to many viral infections.

Cytolysis by CD4⁺ T lymphocytes may not be restricted to cells of lymphoid origin. Although class II MHC antigens are expressed constitutively on the surface of B lymphocytes and macrophages, their expression can be induced in vitro by gamma interferon on a variety of cells, including endothelial cells, fibroblasts, and epithelial cells (19, 23). CD4⁺ clones, when activated, secrete gamma interferon (22, 27)

TABLE 2. Gs lacks hemolysin activity^a

Expt	Antigen	% Hemolysis
1	Intact VSV, 38 μg	41.8
	Gs, 24 μg	7.9
	Buffer A	8.1
2	6-mer peptide, 40 μg	42.2
	Gs, 26 μg	18.3
	Buffer B	17.8

^a Details of the assay have been published (28). Sheep erythrocytes were washed and suspended in buffer at 6×10^7 cells per tube. Virus, peptide, or Gs was added in the indicated amount and incubated for 30 min at 4°C and then for 10 min at 37°C . Intact cells were centrifuged to the bottom, and the supernatant was read at 541 m μ . Results are expressed as a percentage of the absorbency obtained by lysis of the total number of cells in distilled water. Buffer A was McIlvaine buffer, pH 5, and buffer B was 0.01 M acetate at pH 5, both made in physiological saline. The six-amino-acid peptide was synthesized at the Boston Children's Hospital Peptide Facility. Its sequence represents the first six amino acids at the amino terminus of the VSV glycoprotein as defined by Rose and Gallione (26).

and thus may induce class II MHC antigen expression on adjacent cells. It is possible that, at particular times during viral infection, the confluence of soluble glycoprotein(s), antigen-specific CD4⁺ CTLs, and gamma interferon might lead to immunopathology resulting from cell lysis. If this occurs on endothelial cells in the capillary bed, this might contribute to transient viral exanthems. If systemic, this might contribute to the acute hemorrhagic symptoms, leading to death. Since soluble glycoproteins can accumulate in the absence of infectious virus production, such immunopathological processes may occur in the absence of detectable virus.

The ability of APCs to induce proliferation of these CD4⁺ clones and their release of IL-2 supports the idea of an immunoregulatory role. Initially, there is clonal proliferation of these CD4⁺ helper cells. Since they later become CTLs and lyse infected cells and other APCs, the host may use this conversion to avoid uncontrolled expansion of helper CD4⁺ T cells and to shut down the humoral antibody response (2). Viruses may have subverted this normal immunoregulatory mechanism to their own end. If so, the class II MHC-restricted CTLs would be predicted not to be especially protective *in vivo* against viral infections; rather, these CTLs might extend the pathology initiated by the virus. This would be especially so if initially infected cells fail to express class II MHC antigens on their surface and thus are protected from lysis by CD4⁺ CTLs. Studies in progress to evaluate the role of these cells in virus infection following adoptive transfer of CTL clones into naive recipient mice appear to support these predictions (unpublished observations).

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