

## Cell-Mediated Immunity to Herpes Simplex Virus: Specificity of Cytotoxic T Cells

MICHAEL J. P. LAWMAN,<sup>1</sup> RICHARD J. COURTNEY,<sup>1</sup> RICHARD EBERLE,<sup>1</sup> PRICILLA A. SCHAFFER,<sup>2</sup> MICHAEL K. O'HARA,<sup>†</sup> AND BARRY T. ROUSE<sup>1\*</sup>

*Department of Microbiology, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee 37916,<sup>1</sup> and Sidney Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115<sup>2</sup>*

This communication deals with the question of which of the viral antigens constitutes the targets for cytotoxic T lymphocytes (CTL) generated against herpes simplex virus type 1 (HSV-1). The approach used was, first, to compare cytotoxicity of CTL against target cells infected with virus in the presence of tunicamycin and 2-deoxy-D-glucose, which are known to inhibit glycoprotein synthesis, and second, to compare cytotoxicity of CTL against target cells infected with wild-type HSV-1 with that against target cells infected with a temperature-sensitive mutant of HSV-1 which, at the nonpermissive temperature, exhibits diminished glycoprotein synthesis. The results show that glycoprotein expression is required for the demonstration of cytotoxic activity of CTL. The level of cytotoxicity against the temperature-sensitive HSV-1 target at the nonpermissive temperature was reduced and correlated with the level of expression of the major envelope glycoprotein region (VP123; molecular weight = 123,000) at the target cell surface as measured serologically by antibody binding studies. The results were interpreted to indicate that HSV-1-induced glycoproteins are the target antigens for anti-HSV CTL and that the principal viral antigens recognized by the CTL may be glycoproteins of the VP123 region.

The destruction of cells expressing viral antigens by cytotoxic T lymphocytes (CTL) is a well-characterized *in vitro* phenomenon assumed to represent a model for an important *in vivo* mechanism of antiviral defense (4, 9, 48). The observation by Doherty and Zinkernagel that CTL are not only virus specific but that their activity is also restricted by certain gene products of the major histocompatibility complex (the major histocompatibility complex restriction phenomenon) has aroused great interest since the apparent preoccupation of antiviral CTL with major histocompatibility complex antigens may be the driving force for the extreme polymorphism observed within certain regions of the major histocompatibility complex (15, 48). Understanding genetic restriction of antiviral CTL has received considerable attention in recent years, whereas little is known about the molecular identity of viral antigens that engage CTL. This situation is particularly true with complex viruses such as the herpesviruses. With influenza, one of the most extensively studied virus groups, at least two specificities of CTL have been demonstrated, namely, those which are cross-reactive to all type A strains and thought to react with a matrix protein and those which are type-specific and seem to react with

the viral hemagglutinin (7, 18, 50). With ectromelia virus and lymphocytic choriomeningitis virus, the target antigen(s) for CTL is considered to be a glycoprotein (27). The possible nature of the viral antigenic target for CTL induced against herpes simplex virus type 1 (HSV-1) remains unknown, although the knowledge that cells infected with the virus express four or more glycoproteins (43) makes likely the prospect that one or more of these glycoproteins is, in fact, the target antigen. The recent reports of reliable CTL responses against HSV-1 in the mouse species (30, 39), the existence of temperature-sensitive HSV-1 mutants with diminished expression of certain glycoproteins (42), and the availability of inhibitors that selectively prevent the synthesis of certain viral components (13, 31) make possible a study of the HSV-1 viral antigens that are recognized by CTL. The results presented in this communication are interpreted to show that antiherpesvirus CTL react mainly or entirely with viral glycoprotein antigens, and the glycoproteins within the VP123 complex of HSV-1 may be those principally involved.

### MATERIALS AND METHODS

**Cells and viruses.** Strain L-929 cells (*H*-2<sup>d</sup>), obtained from Flow Laboratories, McLean, Va., was used as the syngeneic target cell in all cytotoxicity assays. Strain BALB/c A-31 3T3 cells (*H*-2<sup>d</sup>), obtained from Ray Tennant, Biology Division, Oak Ridge National

<sup>†</sup> Present address: Department of Medical Microbiology, Creighton University School of Medicine, Omaha, NE 68178.

Laboratory, were used as an allogeneic control in the cytotoxicity assays. Both cell lines were grown and maintained in McCoy's 5A medium supplemented with 5% donor calf serum.

Viruses used were HSV-1 strain KOS and a temperature-sensitive mutant of HSV-1 designated *tsA1* (42). The viruses were grown in HEp-2 cells and MRC5 cells (a clone of human embryonic lung cells) based on the method described by Bone and Courtney (6, 9). Titters of virus stocks were determined by plaquing in Vero cells.

**Inhibitors.** Inhibitors of both protein synthesis and glycosylation were employed in this study. The inhibitors of glycosylation were tunicamycin (kindly supplied by Eli Lilly & Co., Indianapolis, Ind.), used at a concentration of 1  $\mu\text{g}/\text{ml}$ , and 2-deoxy-D-glucose (2-DOG) (Calbiochem, La Jolla, Calif.), used at a final concentration of 0.1%. Pactamycin (kindly donated by C. Henney, Fred Hutchinson Cancer Center, Seattle, Wash.) was used as an inhibitor of protein synthesis (46). The optimal concentration of pactamycin was evaluated by measuring the inhibition of the incorporation of [ $^{35}\text{S}$ ]methionine into L cells pretreated with various molar concentrations of pactamycin. The optimal concentration which gave 98% inhibition of protein synthesis was  $10^{-6.5}$  M. The incorporation of pactamycin at this concentration in cytotoxicity assays was noninhibitory to CTL activity.

**Mouse immunization, preparation, and in vitro immunization of spleen cultures.** A detailed account of these procedures was described in a previous communication (30). Briefly, mice (C3H [*H-2<sup>k</sup>*]) were infected intraperitoneally with  $10^{7.0}$  plaque-forming units (0.2-ml inoculum) of wild-type HSV-1. Spleen cell cultures were prepared 4 weeks or more postinfection from HSV-1-immunized mice. For in vitro culture, the spleen cell suspensions (in RPMI 1640 supplemented with a solution of 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, 100  $\mu\text{g}$  of streptomycin per ml, and  $5 \times 10^{-5}$  M 2-mercaptoethanol) were cultured in plastic tissue culture petri dishes at densities of  $2 \times 10^6$  cells per ml and  $1 \times 10^6$  cells per  $\text{cm}^2$  of surface area. The cultures were restimulated (in vitro) with infectious HSV-1 ( $10^{7.0}$  plaque-forming units per ml) for a period of 5 days in a humidified  $\text{CO}_2$  (5%) incubator before measurement of cytotoxicity.

**Cytotoxicity assays.** These were performed essentially as previously described (30). Briefly, L cells were suspended in RPMI 1640-10% fetal calf serum at a cell concentration of  $10^{7.0}/\text{ml}$ , and  $\text{Na}_2^{51}\text{CrO}_4$  (New England Nuclear Corp., Boston, Mass.) was added to a final concentration of 100  $\mu\text{Ci}/\text{ml}$ . Cells were occasionally shaken during the 1-h labeling period, after which the cells were washed twice, and portions were infected at  $37^\circ\text{C}$  with wild-type HSV-1 or *tsA1* for 60 min at a multiplicity of infection of 3. Control cells were not infected. After infection, cells were washed three times in RPMI 1640 at  $37^\circ\text{C}$  and held in RPMI 1640-5% fetal calf serum at either 37, 34, or  $39^\circ\text{C}$  until added to cytotoxicity assays. The procedure was adopted to reduce the chance of expression of the glycoproteins at the nonpermissive temperature by the temperature-sensitive mutant. Target cells,  $10^4$  per well, were added to round-bottom culture plates, and effector cells, at various ratios, were added to the

assay. The assays were run at 37, 34, or  $39^\circ\text{C}$  for 7 h in a humidified  $\text{CO}_2$  incubator. All assays were run in quadruplicate. At the end of incubation, plates were briefly centrifuged, and 50% of the contents was collected for the measurement of radioactivity.

The targets for the inhibitor experiments were prepared differently than the normal targets. L cell monolayers were infected with HSV-1 at a multiplicity of infection of 10 and allowed to adsorb for 1 h at  $37^\circ\text{C}$ . After this period, the cell monolayers were washed once, and McCoy's 5A medium or the same medium containing either tunicamycin (1  $\mu\text{g}/\text{ml}$ ) or 2-DOG (0.1%) was added, and the cell monolayers were incubated for an additional 8 h at  $37^\circ\text{C}$ . The infected cells were removed from the cell culture dish by using 0.1% ethylenediaminetetraacetic acid (EDTA), washed once, and labeled with  $\text{Na}^{51}\text{CrO}_4$  (100  $\mu\text{Ci}/\text{ml}$ ) in media for 1 h at  $37^\circ\text{C}$ . Cells infected in the presence of inhibitors were also labeled in the presence of the same inhibitors. After this time, the cells were washed three times in ice-cold RPMI 1640 before suspension in RPMI 1640 plus 5% fetal calf serum at a cell concentration of  $10^5/\text{ml}$ . The targets were kept on ice until needed. Effector cells were prepared as reported previously (11). These cells were finally suspended in RPMI 1640 plus 5% fetal calf serum containing tunicamycin at 2  $\mu\text{g}/\text{ml}$  and pactamycin at  $2 \times 10^{-6.5}$  M or in medium without additives. Cytotoxicity assays were run either in the presence or absence of the additional inhibitors. Equal volumes of effector and target cells (200- $\mu\text{l}$  total volume per well) were added per well at various effector-to-target cell ratios. In the assays containing inhibitors, the final concentration was 1  $\mu\text{g}$  of tunicamycin per ml and  $10^{-6.5}$  M pactamycin. Quadruplicate determinations of each effector-to-target ratio were performed, and the assays were run for 4 h at  $37^\circ\text{C}$ .

**Computation of specific  $^{51}\text{Cr}$  release.** After the incubation period for the assay (7 or 4 h), 100  $\mu\text{l}$  of the fluid from each well was harvested for measurement of radioactivity. Standard errors were less than 4%, and results were expressed as the mean percent specific  $^{51}\text{Cr}$  release = (effector cell release - medium control release)/(total releasable chromium - medium control release)  $\times 100$ . The total releasable  $^{51}\text{Cr}$  was obtained by exposing target cells to 3% Triton X-100.

**Infection and fixation of monolayers for antibody binding experiments.** L cells were grown in microplates (4 by 6 well) (Costar, Cambridge, Mass.), and confluent monolayers were infected with HSV-1 or *tsA1* at a multiplicity of infection of 10. The virus inoculum was adsorbed for 1 h at  $37^\circ\text{C}$  and washed off, fresh medium was added, and the plates were incubated at 39 or  $34^\circ\text{C}$  for 8 to 9 h postinfection. After incubation, infected and control monolayers were washed once in phosphate-buffered saline (PBS) without serum, and 200  $\mu\text{l}$  of ice-cold 0.2% formaldehyde in PBS was added to each monolayer. The plates were incubated at  $4^\circ\text{C}$  or 1 h, after which monolayers were gently washed three times with PBS before proceeding with the binding experiment. Formaldehyde has previously been shown to have no effect on serologically detectable surface antigens (16) or, in fact, on determinants recognized by cytotoxic T cells (45).

**Procedure for antibody binding experiment.** The cell monolayers, once fixed with formaldehyde

and washed, were reacted with 190  $\mu$ l of rabbit antiserum to either whole HSV-1 or viral glycoproteins or with normal rabbit sera. All sera were diluted in RPMI 1640-5% fetal calf serum and were ultracentrifuged at 100,000  $\times g$  for 1 h to remove aggregates. Monoclonal anti-*H-2<sup>k</sup>* serum produced by clone 11-4.1 (37) obtained from the Salk Institute was purified by affinity chromatography on protein A-Sepharose as described by Herrmann and Mescher (26). This reagent was used to measure the expression of *H-2<sup>k</sup>* glycoproteins. Antisera were left on for 2 h at 4°C. After this period of time, the antisera were removed, and the cell monolayers were washed three times with PBS containing 2% bovine serum albumin. Once the cell monolayers were washed, 200  $\mu$ l of iodinated (<sup>125</sup>I) protein A (kindly prepared by S. Kennel, Oak Ridge National Laboratory) was added and allowed to react for an additional 45 min at 4°C, after which the cell sheets were again washed three times with PBS-bovine serum albumin. After the final wash, the cells were disrupted with 400  $\mu$ l of 0.5 N NaOH and removed from the wells. The wells were then washed with 500  $\mu$ l of PBS, and the total washings were assayed for residual radioactivity on a Beckman 4000 gamma counter. For background counts of nonspecific binding, controls included infected cells reacted with normal rabbit serum and noninfected cells reacted with the specific antisera. Specific binding was expressed by subtracting the counts obtained from the nonspecific binding controls. Preliminary experiments had established that at 8 to 9 h postinfection, levels of antibody binding had reached a maximum level. The dilutions of antisera used were on the plateau of preliminary dose-response curves.

**Preparation of antisera.** The preparation of polypeptide-specific antisera was carried out essentially as described by Courtney and Benyesh-Melnick (11) and Eberle and Courtney (17). The viral glycoprotein region VP123 was purified from HSV-1-infected HEp-2 cells as described previously (R. W. Eberle, Ph.D. thesis, Baylor College of Medicine, Houston, Tex., 1980). Briefly, cells were infected at a multiplicity of infection of 10 plaque-forming units per cell with HSV-1 strain KOS. At 24 h after infection, the cells were harvested and solubilized in 1% sodium deoxycholate and 1% Tween 40 for 1 h at 7°C. The extract was then centrifuged at 100,000  $\times g$  for 1 h, and the solubilized glycoproteins remained in the supernatant. The HSV-specific glycoproteins were separated by two cycles of sodium dodecyl sulfate preparative polyacrylamide gel electrophoresis. The VP123 region (which contains the gA, gB, and gC glycoproteins) and the VP58 region (which contains gD) were collected, concentrated, and prepared for further fractionation and purification by sodium dodecyl sulfate-hydroxylapatite column chromatography according to the procedure of Moss and Rosenblum (33). Two major bands (gC and gA/gB) were resolved from the VP123 region, and two components were also resolved from the VP58 (gD) region. These components were further purified by two additional fractionations through sodium dodecyl sulfate-hydroxylapatite chromatography. The fractions containing the purified glycoproteins were pooled, concentrated, and electrophoresed in cylindrical tube gels. The section of the tube gel containing the purified glycoprotein was cut from the gel and frozen. To

immunize rabbits, gel sections were then thawed and emulsified in Freund complete adjuvant and used according to the procedure previously described (17; Eberle, Ph.D. thesis, Baylor College of Medicine Houston, Tex., 1980).

**Discontinuous polyacrylamide gel electrophoresis.** The details of the method used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis have been previously described (12). Briefly, samples were treated with a solution of sodium dodecyl sulfate (1.0%), urea (0.5 M), and 2-mercaptoethanol (1.0%) for 2 min at 100°C and brought to a 40% sucrose concentration, and 0.2 ml of the sample was electrophoresed at 4 mA per gel until the dye marker (bromophenol blue) had migrated approximately 9.5 cm. The gels were sliced into 2-mm fractions with a gel fractionator (model B100/GMA/GCB; Gilson, Middleton, Wis.) by using a 10% solution of BioSolv (Beckman Instruments, Palo Alto, Calif.) for elution of the isotopically labeled materials from the minced gel fractions. After an overnight incubation, scintillation fluid was added, and the radioactivity was counted on a scintillation counter.

## RESULTS

**Viral glycoprotein synthesis by wild-type and *tsA1* mutant virus-infected cells treated with inhibitors of glycoprotein synthesis.** Herpesvirus glycoproteins are known to be components of the viral envelope and are detectable in all cell membranes as well as at the cell surface of virus-infected cells (25, 34, 38). However, we have previously reported that *tsA1* is a temperature-sensitive mutant which synthesizes greatly diminished quantities of viral glycoproteins at the nonpermissive temperature in human embryonic lung cells (42). To establish if a similar defect could be observed in L-929 fibroblasts used in the cytotoxicity assays, L cells were infected with wild-type HSV-1 and *tsA1* viruses at a multiplicity of infection of 3, cultured at permissive (34°C) and nonpermissive (39°C) temperatures, and isotopically labeled with [<sup>3</sup>H]glucosamine. Levels of glycoprotein synthesis were then determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the cell lysates. As can be seen in Fig. 1, whereas L cells infected with wild-type virus synthesized similar, although not identical, levels of glycoproteins at both 34 (A) and 39°C (C), the *tsA1* virus synthesized markedly reduced levels of glycoproteins at 39°C as compared with those synthesized at the permissive temperature (3°C). The extent of the reduction was greatest with the proteins of the VP123 region (90%) as compared with the synthesis of the glycoproteins of the VP58 region (65%). It is important to note, however, that some glycoproteins of the VP123 region were still being synthesized (approximately 10 to 15%) at the nonpermissive temperature in the L cell system. We have also noted

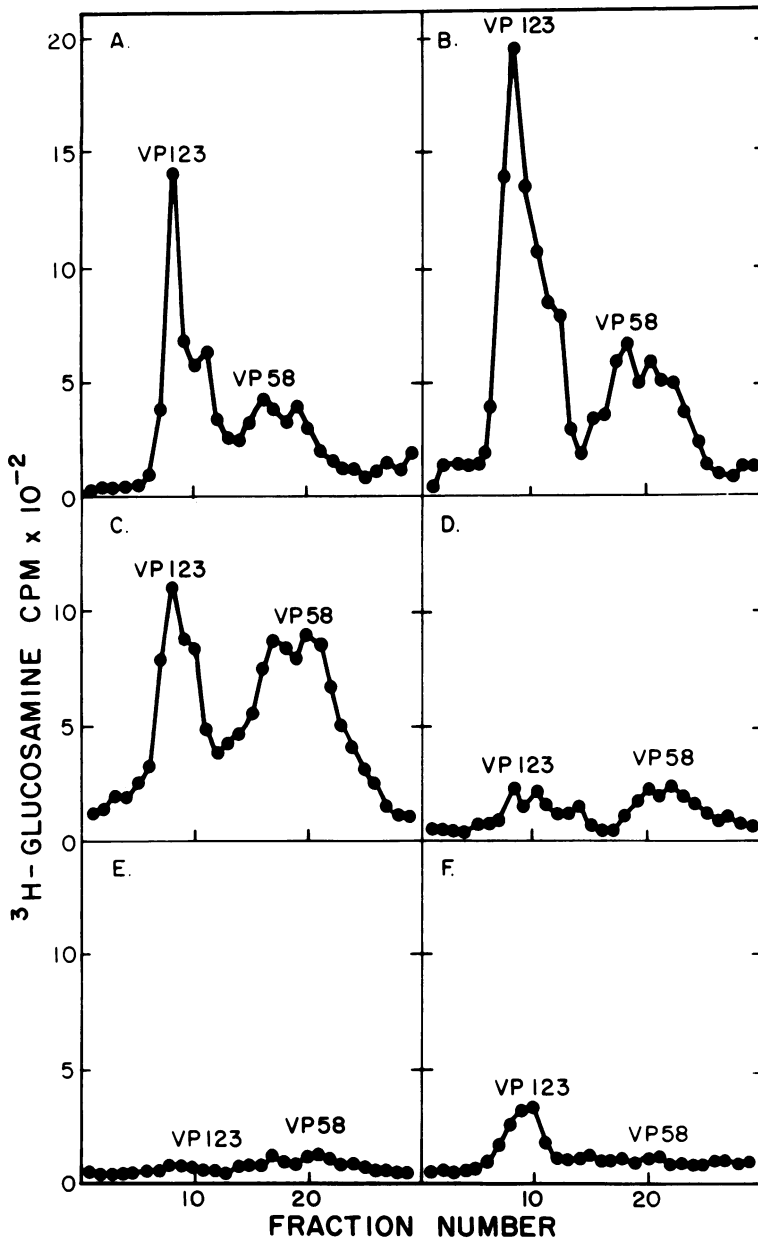


FIG. 1. Acrylamide gel electropherogram of sodium dodecyl sulfate-polyacrylamide gels of [<sup>3</sup>H]glucosamine-labeled proteins. L cells were infected with either HSV-1 wild-type virus or the temperature-sensitive mutant *tsA1* at a multiplicity of infection of 20 plaque-forming units per cell. The cells were maintained at 34 or 39° C in medium containing either 0.1% 2-DOG, 1 μg of tunicamycin per ml, or no inhibitors. All cultures were labeled with [<sup>3</sup>H]glucosamine (10 μCi/ml) from 4 to 24 h postinfection, harvested at 24 h postinfection, and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. After electrophoresis, the middle portion of the gels, which contained the VP123 and VP58 glycoprotein regions, was sliced into 2-mm sections and assayed for radioactivity as described in the text. (A) Wild-type, 34° C; (B) *tsA1*, 34° C; (C) wild-type, 39° C; (D) *tsA1*, 39° C; (E) wild-type plus tunicamycin, 39° C; (F) wild-type plus 2-DOG, 39° C. CPM, Counts per minute.

(data not shown) that the level of reduced glycoprotein synthesis of this mutant in human embryonic lung cells (MRC5) is even greater (90

to 95%) at the nonpermissive temperature compared with that found with L cells.

Shown also in Fig. 1E is evidence that in the

presence of tunicamycin (1  $\mu$ g/ml), synthesis of virus-specific glycoproteins was eliminated. A less marked reduction of glycoprotein synthesis was noted when 2-DOG was used as an inhibitor (Fig. 1F).

**Comparison of the levels of cytotoxicity of CTL against wild-type and *tsA1* virus-infected targets.** We have shown previously that upon infection of mice with HSV-1, specific *H-2*-restricted primary CTL activity develops against target cells infected with wild-type HSV-1 (30). Even higher levels of cytotoxicity were demonstrable if spleen cells from HSV-1-immune mice were cultured in the presence of infectious virus *in vitro* for 5 days before the measurement of their specific cytotoxicity (secondary *in vitro* cytotoxicity) (30). To establish if viral glycoproteins might constitute the target viral antigens for CTL, the activity of CTL generated in mice after primary infection or in secondary *in vitro* stimulation with HSV-1 was measured and compared against target L cells infected with wild-type HSV-1 or *tsA1* at both nonpermissive (39°C) and permissive (34°C) temperatures. The results of a typical experiment (Table 1) indicate that at 39°C, the cytotoxicity of secondary CTL against syngeneic L cell targets infected with the mutant virus was reduced by approximately 50% as compared with

the cytotoxicity directed against wild-type virus-infected target cells (35% compared with 68% specific  $^{51}\text{Cr}$  release). However, the levels of cytotoxic activity against wild-type and mutant virus-infected targets at the permissive temperature (34°C) were similar; the cytotoxic activity against *tsA1*-infected cells was 90% of that directed against the wild-type virus-infected target. A similar pattern of results was observed in three repeat experiments. We have also shown that the activity of primary CTL against *tsA1*-infected targets at 39°C was similarly reduced by approximately 50% of that against wild-type targets (data not shown). As reported previously (30), levels of cytotoxicity against allogeneic virus-infected cells did not exceed that against uninfected L cells. Thus, the response being measured was *H-2* restricted and presumably T cell in nature.

**Effect of inhibitors of glycosylation on levels of cytotoxicity.** Comparisons of the levels of cytotoxicity were made between target cells infected with wild-type virus and targets infected with wild-type virus in the presence of 2-DOG and tunicamycin, which are known inhibitors of glycosylation (13, 31). In these experiments, the cytotoxic activity of CTL was measured both in the absence of inhibitors and in the presence of tunicamycin and pactamycin in the medium during the assay. The rationale for the addition of tunicamycin and pactamycin during the assay was to prevent further *de novo* protein synthesis and, therefore, the subsequent production of glycosylated proteins from occurring during the time of the assay (4 h).

The presence of tunicamycin and pactamycin at the concentration used had no inhibitory effect on CTL activity and, in fact, in the experiment shown in Table 2, the level of cytotoxicity in the presence of the drugs was slightly elevated. In each of the five experiments conducted to determine the effects of inhibitors of glycosylation on HSV-1-infected target cells, a marked reduction in cytotoxic activity was observed. The extent of the reduction varied somewhat, although the experimental conditions remained constant. However, as shown in Table 2, the following features were always apparent: (i) the reduction in cytotoxicity observed was always greater against tunicamycin-treated target cells than 2-DOG-treated target cells; and (ii) the reduction of cytotoxic activity was more marked against the drug-treated target cells when tunicamycin and pactamycin were included during the cytotoxicity assay. A representative experiment shown in Table 2 indicates that cytotoxic activity against target cells infected in the presence of tunicamycin was inhibited by 90 to 100% when additional drugs

TABLE 1. Cytotoxicity of *in vitro* HSV-immunized spleen cells<sup>a</sup> against wild-type HSV-1 strain KOS and *tsA1* virus-infected target cells at different temperatures

Temp (°C)	Effector/target ratio	% Specific $^{51}\text{Cr}$ release <sup>b</sup>		
		L cells + HSV-1 WT <sup>c</sup>	L cells + <i>tsA1</i>	L cells
34	25:1	53.0	48.2	7.2
	12:1	41.3	40.0	6.0
	6:1	14.2	16.1	5.0
	3:1	7.0	6.1	2.1
39	25:1	68.0	35.0	6.7
	12:1	50.7	27.2	4.7
	6:1	32.1	11.3	5.0
	3:1	17.2	5.2	2.0

<sup>a</sup> Spleen cells from C3H mice infected 6 weeks previously with HSV-1. Cells were cultured for 5 days *in vitro* with HSV-1 before use in the cytotoxicity assay.

<sup>b</sup> Computed as described in the text. Assays were performed for 7 h. The standard errors of the mean of quadruplicate determinations did not exceed 4%. The background release from target cells during assays for L-HSV-1, L-HSV-1 *tsA1*, and uninfected L cells was 31, 33, and 28%, respectively. Not shown in the table is that cytotoxicity against A-31 3T3 (*H-2*<sup>d</sup>) cells infected with HSV-1 or *tsA1* was approximately the same as that against uninfected L cells.

<sup>c</sup> WT, Wild-type.

TABLE 2. Effect of tunicamycin and 2-DOG on susceptibility of HSV-1-infected target cells to cytotoxicity<sup>a</sup>

Assay conditions	% specific <sup>51</sup> Cr release				
	Effector/target ratio	L-HSV-1	L-HSV-1 + tunicamycin	L-HSV-1 + 2-DOG	L cells
Inhibitors not present during assay	40:1	76.1	33.8 (63) <sup>b</sup>	55.0 (31)	8.8
	20:1	67.0	29.6 (63)	49.1 (30)	7.0
	10:1	56.1	22.0 (67)	45.2 (22)	5.2
	5:1	44.2	15.9 (63)	31.8 (30)	2.8
Inhibitors present during assay <sup>c</sup>	40:1	84.9	16.3 (90)	31.1 (70)	8.1
	20:1	80.0	6.9 (100)	30.3 (68)	6.9
	10:1	71.0	2.8 (100)	24.1 (73)	6.7
	5:1	57.9	1.4 (100)	16.9 (75)	3.2

<sup>a</sup> Assays were performed for 4 h. The target cells were infected at a multiplicity of infection of 10, 8 h before commencement of assays. The standard errors for the quadruplicate determinations did not exceed 4%.

<sup>b</sup> The numbers within parentheses are percent inhibition values. These were computed by subtracting L cell background from the virus-infected targets and then expressing the cytotoxicity against drug-treated targets as a percentage of that against targets with no inhibitor present.

<sup>c</sup> Tunicamycin, 1 µg/ml; pactamycin, 10<sup>-6.5</sup> M.

were present during the assay. However, when 2-DOG was used as an inhibitor, the reduction in cytotoxic activity ranged from 68 to 75%. When inhibitors were not included during the cytotoxicity assay, the levels of inhibition ranged from 63 to 67% against tunicamycin-treated targets and 22 to 31% against 2-DOG-treated targets.

Since tunicamycin prevents glycosylation of all proteins, the observed reduced cytotoxicity of tunicamycin-treated virus-infected targets could have been explained by a failure of synthesis and expression of either viral glycoproteins, *H-2* glycoproteins, or both. However, two lines of evidence favored the hypothesis that the effect was mainly on viral glycoprotein expression. Thus, we have observed that L cells treated with tunicamycin under the same conditions as described in the virus cytotoxicity assays were still susceptible to cytotoxic destruction by BALB/c anti-*H-2*<sup>k</sup> CTL (Table 3). There was some reduction in this alloctotoxicity, but this reduction was insufficient to account for the extent of reduced antiviral cytotoxicity observed against tunicamycin-treated virus-infected targets. Unfortunately, the experimental design does not permit an assessment as to whether or not the elimination of antiviral cytotoxicity was associated with the effects of tunicamycin on both virus and *H-2* glycoprotein expression. The second line of evidence that target cells treated with tunicamycin still express *H-2* glycoproteins is presented below.

**Antibody binding to virus-infected cells.** It is apparent from the work of others that CTL react with antigens present at the cell surface (14, 20, 45). Furthermore, it has been shown that the protein moiety of herpesvirus glycoproteins

TABLE 3. Cytotoxic activity of BALB/c anti-*H-2*<sup>k</sup> effector cells against HSV-1-infected and uninfected L cells and against these same targets treated with tunicamycin<sup>a</sup>

Assay conditions	Effector/target ratio <sup>b</sup>	% Specific lysis <sup>c</sup>			
		L cells	L cells + tunicamycin	L-HSV	L-HSV + tunicamycin
Inhibitors present <sup>d</sup>	40	86.8	76.0	81.0	71.4
	20	78.6	73.0	78.9	67.8
	10	68.9	60.1	65.3	51.0
Inhibitors not present	40	79.2	65.0	73.5	67.2
	20	78.3	62.3	70.0	63.9
	10	68.0	53.9	65.5	59.2

<sup>a</sup> L cells and virus-infected cells were treated with 1 µg of tunicamycin per ml for 8 h, after which they were labeled with <sup>51</sup>Cr for 1 h in the presence of tunicamycin.

<sup>b</sup> BALB/c spleen cells from mice previously immunized against *H-2*<sup>k</sup> were cultured in vitro for 5 days with mitomycin C-treated *H-2*<sup>k</sup> spleen cells.

<sup>c</sup> Assay time, 4 h.

<sup>d</sup> Tunicamycin (1 µg/ml) and pactamycin (10<sup>-6.5</sup> M) were included during the 4-h assay.

constitutes the antigenic components to which antibody production is directed (R. J. Courtney and E. Wenske, unpublished data). Although T cells often recognize different antigens than those detected serologically (1, 32) the likelihood that anti-HSV T cells also react with proteins rather than carbohydrate determinants seems high. Thus, our observation that glycoprotein synthesis, as measured in sodium dodecyl sul-

fate-polyacrylamide gel electrophoresis profiles of labeled carbohydrate components, was reduced by approximately 90% in mutant virus-infected cells does not necessarily mean that these infected cells do not express some glycoprotein or, alternatively, some nonglycosylated protein of the same antigenicity at the cell surface. It is possible that even low levels of glycoprotein expression at the cell surface may permit some cytotoxicity to occur. To measure the expression of herpesvirus glycoprotein antigens at the surface of virus-infected cells, these cells were reacted with antisera specific for glycoproteins gA/gB and gC of the VP123 glycoprotein complex of HSV-1 (10). As shown in Fig. 2, at the permissive temperature, the binding of anti-gA/gB and anti-gC to wild-type and *tsA1* virus-infected cells was similar. However, at the non-permissive temperature (39°C), binding to *tsA1*-infected cells by both antisera was reduced, although not eliminated. In fact, the extent of binding to *tsA1*-infected cells at 39°C was about 50% (55% with anti-gC and 35% for anti-gA/gB [Fig. 2]) of that to wild-type virus-infected cells. We also observed approximately 50% binding with anti-VP123 (data not shown). However, with antisera to VP58 (gD), we noted no significant reduction in binding to *tsA1*-infected cells at 39°C.

Also shown in Fig. 2 are results of binding experiments with tunicamycin and 2-DOG-treated infected cells. It is readily apparent that the extent of binding of anti-gA/gB and anti-gC to infected cells treated with tunicamycin was barely detectable (90 to 98% inhibition) in comparison with binding to non-drug-treated infected cells. However, the reduction in antigen expression in infected cells treated with 2-DOG was apparently less. In this case, the level of inhibition was 35% for anti-gC and 45% for anti-gA/gB.

Binding experiments were also performed with tunicamycin-treated infected and uninfected L cells with anti-*H-2<sup>k</sup>* antisera to evaluate the expression of *H-2* glycoproteins. In comparison with the binding of anti-*H-2<sup>k</sup>* serum to uninfected, untreated L cells, the following reductions in binding were observed (average of two experiments): tunicamycin-treated uninfected L cells, 34.7%; tunicamycin-treated virus-infected L cells, 41.6%; and virus-infected L cells, 23.9%. Thus, under the conditions used in our experiments, tunicamycin failed to eliminate the expression of *H-2* glycoproteins.

## DISCUSSION

The observation that CTL destroy target cells by reacting with antigens expressed at the sur-

face of target cells has been well established (9, 28). However, with the possible exception of CTL reacting with haptens such as trinitrophenyl groups (22), the identity of the molecular species that is recognized by CTL has not been established. With viral antigens, the best clues as to antigen specificity of CTL have been documented with the influenza viruses and vesicular stomatitis virus (7, 8, 18, 19, 49, 50). Against influenza, at least two CTL specificities were demonstrated, one of which is considered to react with common determinants on the hemagglutinin (29) or with a matrix antigen found internally in virions but expressed at the surface of cells infected by all type A influenza viruses (8, 18, 50). The other population of CTL generated against influenza is strain specific and is presumed to react with the viral hemagglutinin (16, 49). With vesicular stomatitis virus, the situation is more simple than with influenza since infection by vesicular stomatitis virus leads to the expression of only two well-defined antigens associated with cell membranes; a matrix protein (M) and a glycoprotein (G) (40, 47). Since temperature-sensitive mutants exist which fail to express either the M or G antigens, it was possible to demonstrate that the target antigen for vesicular stomatitis virus CTL was the glycoprotein alone (24). The identity of the target antigen(s) for CTL directed against HSV-1, a virus group not previously investigated, constitutes the subject of the present report. We have interpreted our results to indicate that HSV-1-induced glycoproteins are the target antigens and that glycoproteins of the VP123 complex are perhaps the principal antigens involved in CTL recognition. Two experimental approaches were used to arrive at these conclusions; (i) to study the effect on cytotoxicity of drugs that inhibit glycoprotein synthesis and (ii) to compare the susceptibility to lysis of cells infected with a temperature-sensitive mutant impaired in glycoprotein synthesis at the nonpermissive temperature with that of wild-type virus-infected cells at this temperature.

In the first approach, two drugs were chosen to inhibit glycoprotein synthesis, tunicamycin and 2-DOG. The former drug, an antibiotic, was shown previously to markedly diminish HSV-1 glycoprotein synthesis, while at the same time not to affect protein synthesis (R. J. Courtney, unpublished data). In fact, infected cells may still synthesize proteins antigenically similar or identical to the glycoproteins of untreated cells, although these proteins are not transported or not incorporated into cell membranes as in the case of glycosylated proteins (E. Wenske and R. J. Courtney, unpublished data). In the present

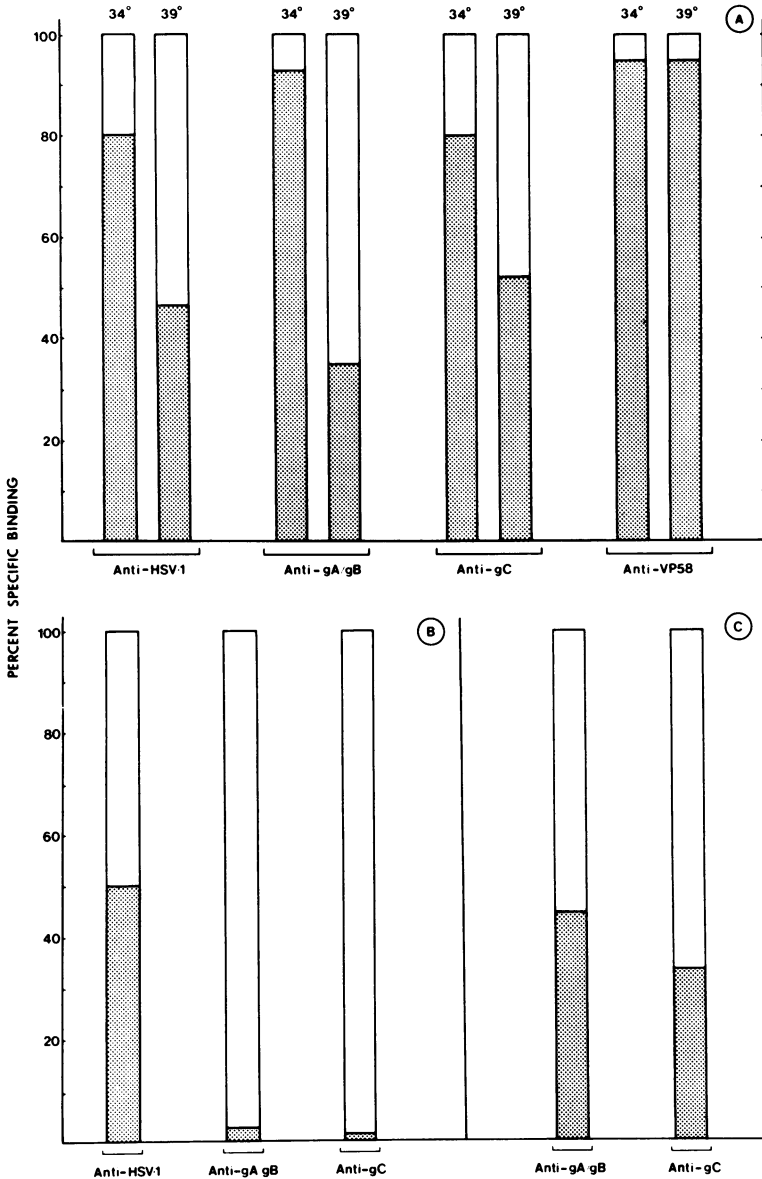


FIG. 2. Demonstration of herpesvirus antigen expression on the surface of virus-infected cells by binding of antisera to HSV-1 and to virus-specific glycoproteins. (A) The percent binding of antisera to tsA1-infected cells (stippled area) relative to wild-type virus-infected cells at both permissive (34° C) and nonpermissive (39° C) temperatures. (B) The percent binding of antisera to tunicamycin-treated virus-infected cells (stippled area) relative to untreated wild-type virus-infected cells. (C) The percent binding to antisera to 2-DOG-treated virus-infected cells (stippled area) relative to untreated wild-type virus-infected cells.

report, we show that tunicamycin-treated HSV-1-infected L cells failed to synthesize glycoproteins as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of the [<sup>3</sup>H]glucosamine-labeled proteins. Furthermore, when tunicamycin-treated cells were examined for the expression of certain viral glycoprotein

antigens by their ability to bind specific antisera, these antigens were found lacking. The tunicamycin-treated L cell was therefore a useful target cell for assessing the role of viral glycoproteins as target antigens for CTL. Our experiments revealed a marked reduction or total abolition of cytotoxicity against inhibited cells, pro-



vided that steps were taken to prevent glycoprotein synthesis during the cytotoxicity assay. This was achieved by including tunicamycin and pactamycin in the assay. These drugs were noninhibitory to the activity of CTL as revealed by control experiments; in fact, cytotoxicity in the presence of both drugs was sometimes slightly enhanced. The rationale for the use of the drugs was that the tunicamycin would prevent glycosylation of proteins already synthesized, whereas pactamycin would prevent any further protein synthesis in infected cells (46). When the drugs were not included during the assay, some cytotoxicity, approximately 30% of control values, was observed.

The glucose-mannose analog 2-DOG was also used as an inhibitor to assess the role of glycoproteins in anti-HSV CTL activity. With this drug, synthesis of altered glycoproteins and a nonglycosylated precursor antigenically similar to normal glycoproteins occurs (31; E. Wenske and R. J. Courtney, unpublished data). Some cytotoxic activity against 2-DOG-inhibited target cells was always observed, especially when tunicamycin and pactamycin were not included during the cytotoxicity assay. However, it is well known that the effects of 2-DOG are rapidly reversible (31) and, consequently, glycoprotein synthesis could have proceeded normally, thus providing the target antigens for cytotoxicity during the 4-h assay. When further glycoprotein synthesis was prevented during the assays by inclusion of tunicamycin and pactamycin during the assay, the level of inhibition of cytotoxicity was about 70%—a result similar to that observed with 2-DOG inhibition of CTL against ectromelia virus-infected cells (27). This level of inhibition also corresponded closely with the reduction in expression of serologically detectable glycoproteins at the cell surface of 2-DOG-treated virus-infected cells.

Taken together, the results of studies on the effects of glycosylation inhibitors on CTL activity provide strong evidence that viral glycoprotein expression is essential for lysis. However, the approach with inhibitors does not reveal the nature of the glycoprotein molecule(s) recognized by CTL. Further insight into this problem was obtained from experiments in which the cytotoxicity of CTL against wild-type virus-infected cells was compared with that against cells infected with a temperature-sensitive mutant which, at the nonpermissive temperature, synthesized reduced quantities of glycoproteins, especially glycoproteins of the VP123 complex. When the levels of cytotoxic activity by CTL against wild-type and temperature-sensitive mutant virus-infected cells were compared, approximately 50% inhibition of activity was observed

against the temperature-sensitive mutant virus-infected targets at the nonpermissive temperature. A similar reduction was also observed in expression of the glycoproteins in the VP123 complex as measured serologically in antibody binding assays. However, with antisera specific to glycoprotein VP58 (gD), little or no reduction in VP58 antigen expression was observed. These data together indicate that the target antigens for anti-HSV-1 CTL are glycoproteins and principally those of the VP123 complex. However, certain cautionary points should be made. First, the shutoff of glycoprotein synthesis by the temperature-sensitive mutant was not absolute, and the number of molecules capable of inducing susceptibility to lysis by CTL is not known. Thus, all cells may synthesize sufficient VP123 glycoproteins to permit lysis by CTL directed against these proteins, and the reason for impaired cytotoxicity may lie elsewhere, such as in the expression of an unrelated antigen or a change in the nature of the association of the actual target antigen with *H-2* antigens. For example, it is widely accepted that recognition by CTL involves both *H-2* antigen as well as viral antigen (*H-2* restriction), and, if the single receptor hypothesis (15) is true, perhaps lysis of the mutant target is diminished because target antigens do not interact optimally with *H-2* components in the case of the mutant virus.

The second point of caution is that in most, if not all, T cell cytotoxicity systems investigated, antigens detected by CTL are not identical to those measured serologically (1, 21, 23, 32). Thus, for example, in the extensively studied allogeneic system, CTL are often considered to react with "lymphocyte-determined" antigens, and antibody is considered to react with "serologically determined" antigens (2, 44). In fact, whereas T cells of the cytotoxic type can distinguish mutants of the K allele of the major histocompatibility complex K region, antibody cannot (32). Whether or not the antigen detectable by CTL and antibody are on the same molecule or represent close but distinct neighbors in the plasma membrane has not been resolved. Such observations may mean that our measurement of a 50% reduction in cytotoxicity against the temperature-sensitive mutant and a corresponding 50% reduction in the expression of VP123 glycoproteins measured serologically does not imply causality.

The resolution of these cautionary points will require investigations with additional mutants, preferably those which do not express individual glycoproteins, as well as other approaches, such as the use of specific antisera to inhibit cytotoxicity and cold-target competition experiments. Such approaches are currently underway in our

laboratory. However, that VP123 glycoproteins rather than those in the VP58 region may provide the principal targets can be assumed circumstantially from studies on the specificity of CTL. For example, Pfizenmaier et al. (39) have shown that CTL mounted against HSV-1 are specific to that virus and do not cross-react with HSV-2. Similar results have been recorded with *in vitro* immunized CTL (R. Eberle, M. J. P. Lawman, and B. T. Rouse, unpublished data), namely, that cells highly cytotoxic to HSV-1 targets show minimal cytotoxic activity against syngeneic HSV-2-infected targets. Since glycoproteins of the VP58 region of HSV-1 and HSV-2 are immunologically related (10), 35; R. J. Courtney, unpublished data), this makes it unlikely that VP58 is the principal target glycoprotein for HSV-1 CTL activity.

If it is shown ultimately that VP123 glycoproteins, or perhaps one or more of the three glycoprotein subcomponents of VP123 (25, 43), constitute the target antigen for CTL, our results will have implications for the development of future subunit vaccines. Thus, it has already been shown that glycoproteins in the VP123 complex provide the principal immunogens responsible for eliciting neutralizing antibody production (22). If the same glycoproteins are shown to induce the parameters of cell-mediated immunity, the mechanism considered by many to be most crucial for resolution of herpes infections (5, 36, 41), then any subunit vaccine for use against HSV-1 should be minimally composed of these glycoproteins.

#### ACKNOWLEDGMENTS

Financial assistance was provided by Public Health Service grants A1 14981 and CA 24564 from the National Institutes of Health.

We thank Mary Alford and Beth Bowling-Ethier for excellent technical assistance.

#### LITERATURE CITED

- Alkan, S. S., D. E. Nitecki, and J. W. Goodman. 1971. Antigen recognition and the immune capacity of L-tyrosine-azobenzenesulfonate to serve as a carrier for macromolecular haptens. *J. Immunol.* **107**:353-360.
- Bach, F. H. 1976. Differential function of MHC, LD and SD determinants, p. 417. *In* V. P. Eijsvoogel, D. Roos, and W. P. Zeijlemaker (ed.), *Proceedings of the 10th Leukocyte Culture Conference*. Academic Press, Inc., New York.
- Ballas, Z. K., and C. S. Henney. 1979. Generation of H-2 restricted cytotoxic T cells by trinitrophenylated proteins *in vitro*: specificity and requirements. *J. Immunol.* **123**:1696-1704.
- Blanden, R. V. 1974. T cell response to viral and bacterial infection. *Transplant. Rev.* **19**:56-88.
- Bloom, B. R., and B. R. Zisman. 1975. Cell mediated immunity in viral infections, p. 113-136. *In* A. L. Notkins (ed.), *Viral immunology and immunopathology*. Academic Press, Inc., New York.
- Bone, D. R., and R. J. Courtney. 1974. A temperature sensitive mutant of herpes simplex virus type 1 defective in the synthesis of the major capsid polypeptide. *J. Gen. Virol.* **24**:17-27.
- Braciale, T. 1977. Immunologic recognition of influenza virus infected cells. I. Generation of a virus strain specific and cross reactive subpopulation of cytotoxic T cells in response to type A influenza viruses of different subtypes. *Cell. Immunol.* **33**:423-436.
- Braciale, T. 1977. Immunologic recognition of influenza virus infected cells. II. Expression of influenza A matrix protein on the cell surface and its role in recognition by cross reactive cytotoxic T cells. *J. Exp. Med.* **146**:673-680.
- Cerottini, J. C., and K. T. Brunner. 1974. Cell mediated cytotoxicity, allograft rejection and tumor immunity. *Adv. Immunol.* **18**:67-132.
- Cohen, G. H., M. Katze, C. Hydrean-Stern, and R. J. Eisenberg. 1978. Trypan-blue CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular-weight envelope glycoprotein. *J. Virol.* **27**:172-181.
- Courtney, R. J., and M. Benyesh-Melnick. 1974. Isolation and characterization of a large molecular weight polypeptide of herpes simplex virus type 1. *Virology* **62**:539-551.
- Courtney, R. J., R. M. McCombs, and M. Benyesh-Melnick. 1971. Antigens specified by herpesvirus. II. Effect of arginine deprivation in the synthesis of cytoplasmic and nuclear proteins. *Virology* **43**:356-365.
- Courtney, R. J., S. M. Steiner, and M. Benyesh-Melnick. 1973. Effects of 2-deoxy-D-glucose on herpes simplex virus replication. *Virology* **52**:447-455.
- Dennert, G. 1976. Thymus derived killer cells: specificity of function and antigen recognition. *Transplant. Rev.* **29**:59-88.
- Doherty, P. C., R. V. Blanden, and R. M. Zinkernagel. 1976. Specificity of virus immune effector T cells for H-2K or H-2D compatible interactions. I. Implications for H-antigen diversity. *Transplant. Rev.* **29**:89-124.
- Drake, W. P., P. C. Ungaro, and M. R. Mardiney. 1972. Preservation of cellular antigenicity of tumor cells by the use of Formalin fixation. *Cancer Res.* **32**:1042-1046.
- Eberle, R., and R. J. Courtney. 1980. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. *J. Virol.* **35**:902-917.
- Effros, R. B., P. C. Doherty, W. Gerhard, and J. Bennick. 1977. Generation of both cross-reactive and virus specific T cell populations after immunization with serologically distinct influenza A viruses. *J. Exp. Med.* **145**:557-567.
- Ennis, F. A., W. J. Martin, and M. W. Verbonitz. 1977. Hemagglutinin specific cytotoxic T cells during influenza infection. *J. Exp. Med.* **146**:893-898.
- Forman, J. 1976. The specificity of thymus derived T cells in cell mediated cytotoxic reactions. *Transplant. Rev.* **29**:146-163.
- Gell, P. G. H., and B. Benacerraf. 1959. Studies on hypersensitivity to denatured protein in guinea pigs. *Immunology* **2**:64-74.
- Glorioso, J. C., L. A. Wilson, T. W. Fenger, and J. W. Smith. 1978. Complement-mediated cytolysis of HSV-1 and HSV-2 infected cells: plasma membrane antigens reactive with type-specific and cross-reactive antibody. *J. Gen. Virol.* **40**:443-454.
- Gooding, L. R. 1977. Antibody blockade of lysis by T lymphocyte effectors generated against syngeneic SV40 transformed cells. *J. Immunol.* **122**:2328-2336.
- Hale, A. H., N. W. Owen, D. Baltimore, and H. N. Eisen. 1978. Vesicular stomatitis virus glycoprotein is necessary for H-2 restricted lysis of infected cells by cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **75**:970-974.

25. Heine, J. W., P. G. Spear, and B. Roizman. 1972. Proteins specified by herpes simplex virus. VI. Viral proteins in the plasma membrane. *J. Virol.* **9**:431-439.
26. Herrmann, S. H., and M. F. Mescher. 1979. Purification of the  $H-2^k$  molecule of murine major histocompatibility complex. *J. Biol. Chem.* **254**:8713-8716.
27. Jackson, D. C., G. L. Ada, A. J. Hapel, and M. B. C. Dunlop. 1976. Changes in the surface of virus-infected cells recognized by cytotoxic T cells. *Scand. J. Immunol.* **5**:1021-1029.
28. Janeway, C. A. 1976. The specificity of T lymphocytic responses to chemically defined antigens. *Transplant. Rev.* **29**:164-188.
29. Kozinowski, U. H., H. Allen, M. Gething, M. D. Waterfield, and H. Klenk. 1980. Recognition of viral glycoproteins by influenza A-specific cytolytic T lymphocytes. *J. Exp. Med.* **151**:945-958.
30. Lawman, M. J. P., B. T. Rouse, R. J. Courtney, and R. D. Walker. 1980. Cell-mediated immunity against herpes simplex induction of cytotoxic T lymphocytes. *Infect. Immun.* **27**:133-139.
31. Leavitt, R., S. Schlesinger, and S. Kornfeld. 1977. Tunicamycin inhibits glycosylation and multiplication of Sindbis and vesicular stomatitis virus. *J. Virol.* **21**:375-385.
32. McKenzie, I. F. C., T. Pang, and R. V. Blanden. 1977. The use of H-2 mutants as models for study of T cell activation. *Immunol. Rev.* **35**:181-230.
33. Moss, B., and E. W. Rosenblum. 1972. Hydroxylapatite chromatography of protein-sodium dodecyl sulfate complexes. I. A new method for the separation of polypeptide subunits. *J. Biol. Chem.* **247**:5194-5198.
34. Norrild, B. H., H. Ludwig, and R. Roth. 1978. Identification of a common antigen of herpes complex virus, bovine herpes mammillitis virus, and B virus. *J. Virol.* **26**:712-717.
35. Norrild, B., and B. F. Vestergaard. 1977. Polyacrylamide gel electrophoretic analysis of herpes simplex virus type 1 immunoprecipitates obtained by quantitative immunoelectrophoresis in antibody-containing agarose gel. *J. Virol.* **22**:113-117.
36. Notkins, A. L. 1974. Commentary: immune mechanisms by which the spread of viral infection is stopped. *Cell. Immunol.* **11**:478-483.
37. Oi, V., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig antigens, H-2 and Ia antigens. *Curr. Top. Microbiol. Immunol.* **81**:115-129.
38. Olshevsky, U., and Y. Becker. 1972. Surface glycopeptides in the envelope of herpes simplex virions. *Virology* **50**:277-279.
39. Pfizenmaier, K., H. Jung, A. Powtz, M. Pollinghoff, and H. Wagner. 1977. The role of T cells in antiherpes simplex virus immunity. I. Induction of antigen specific cytotoxic T lymphocytes. *J. Immunol.* **119**:939-944.
40. Pringle, C. R. 1977. Genetics of rhabdoviruses, p. 239-287. *In* H. F. Conrat and R. R. Wagner (ed.), *Comprehensive virology*, Plenum Publishing Corp., New York.
41. Rouse, B. T., and L. A. Babiuk. 1978. Mechanisms of recovery from herpesvirus infections—a review. *Can. J. Comp. Med.* **42**:414-427.
42. Schaffer, P. A., R. J. Courtney, R. M. McCombs, and M. Benyesh-Melnick. 1971. A temperature-sensitive mutant of herpes simplex virus defective in glycoprotein synthesis. *Virology* **46**:356-368.
43. Spear, P. G. 1976. Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type 1-infected cells. *Virology* **17**:991-1008.
44. Stulberg, R., R. Todd, and L. Gooding. 1976. Susceptibility of anti H-D2 capped target cells to humoral and T lymphocyte-mediated lysis. *Transplantation* **21**:71-80.
45. Stulting, R. D., R. F. Todd, and D. B. Amos. 1975. Lymphocyte mediated cytotoxicity against fetal fibroblasts II isolation and target cell specificity of the effector cells. *Cell. Immunol.* **40**:69-76.
46. Thorn, R. M., and C. S. Henney. 1976. Studies on the mechanism of lymphocyte-mediated cytolysis. VI. A reappraisal of the requirement for protein synthesis during T cell-mediated lysis. *J. Immunol.* **116**:146-149.
47. Wagner, R. R. 1975. Reproduction of rhabdoviruses, p. 1-94. *In* H. F. Conrat and R. R. Wagner (ed.), *Comprehensive virology*, Plenum Publishing Corp., New York.
48. Zinkernagel, R. M. 1978. Speculations on the role of major transplantation antigens in cell mediated immunity against intracellular parasites. *Curr. Top. Microbiol. Immunol.* **82**:113-138.
49. Zweerink, H. J., B. A. Askonas, D. Millican, S. A. Courtneidge, and J. J. Skehel. 1977. Cytotoxic T cells to type A influenza virus. I. Virus hemagglutinin induces A strain specificity while infected cell confer cross reactive cytotoxicity. *Eur. J. Immunol.* **7**:630-635.
50. Zweerink, H. J., S. A. Courtneidge, J. J. Skehel, M. J. Crumpton, and B. A. Askonas. 1977. Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. *Nature (London)* **267**:354-356.