INFECTION AND IMMUNITY, July 1982, p. 116–126
0019-9567/82/070116-11$02.00/0

Vol. 37, No. 1

Intratypic and Intertypic Specificity of Lymphocytes Involved in the Recognition of Herpes Simplex Virus Glycoproteins

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Received 3 December 1981/Accepted 9 March 1982

Cytotoxic T lymphocytes (CTL) were generated in C57BL/6 mice with herpes simplex virus type 1 (HSV-1) (strains KOS, 17, HFEM, and mP) and HSV-2 (strains 186, G, and GP6). Effector lymphocytes were tested for cytotoxicity against syngeneic HSV-1- and HSV-2-infected cells in a 5-h ^{51}Cr release assay. HSV-1 strain HFEM was found to induce CTL efficiently only when 100-fold more virus was used as compared with HSV-1 strains KOS, 17, and mP. All HSV-1 and HSV-2 strains induced cross-reactive populations of CTL. CTL generated by HSV-1 KOS and HSV-2 186 also demonstrated cross-reactivity in an ear-swelling model for delayed-type hypersensitivity. Lymphocytes generated by all HSV-2 strains were highly efficient at lysing HSV-1-infected target cells. However, HSV-2-infected target cells were found to be less susceptible to lysis by either HSV-1 or HSV-2 CTL than were HSV-1-infected target cells. The lowered susceptibility of HSV-2-infected cells was not due to an inefficient infection of BL/6 WT-3 cells as measured by standard growth assays and infectious center assays. Varying the multiplicity of infection or the time of infection did not increase the susceptibility of HSV-2-infected target cells to lysis by CTL. Increasing the effector-to-target-cell ratio resulted in an increased lysis of both HSV-1- and HSV-2-infected target cells by CTL, but the level of HSV-2-infected target cell lysis still did not approach the level of HSV-1-infected target cell lysis. HSV-2-infected cells were as efficient as HSV-1-infected cells in the cold cell competition assay employed in reducing the lysis of ^{51}Cr-labeled, HSV-1-infected target cells. In addition, HSV-2-infected cells were susceptible to lysis by HSV-immune serum and complement.

Host responses to herpes simplex virus (HSV) involve both humoral and cell-mediated immune mechanisms. Cytotoxic thymus-derived (T) cells and delayed-type hypersensitivity (DTH) cells are involved in the rapid clearance of HSV and protection against acute HSV infections in mice in vivo (20, 28–33, 44), and HSV-specific cytotoxic T cells have been shown to lyse syngeneic HSV-infected target cells in vitro (5, 24, 39, 40, 50).

Two major serological types of HSV (HSV-1 and HSV-2) have been identified, and the DNAs can be separated by restriction enzyme analysis (48). Different isolates (strains) of either virus type can also be differentiated by restriction enzyme analysis (21, 26). Serological studies have shown that cross-reactive antibodies are induced in the host after infection with either HSV-1 or HSV-2 (41, 52, 53). The virus-specific glycoproteins function in the induction of both humoral and cell-mediated responses to HSV (5, 15, 24, 34, 35). Both type-common and type-specific antigenic determinants have been identified on glycoproteins gA/gB, gD, and gE (2, 19, 35, 36, 54). Currently, only type-specific determinants have been identified on gC (54, 56). Several strains of HSV-1 and HSV-2 have been examined in individual studies of both cytotoxic T cell induction and reactivity (5, 24, 33, 39, 40, 50) and the DTH response to HSV infection (22, 28–33, 47). Both type-specific (39, 40) and cross-reactive responses (50, 51) in the cytotoxic T cell assay have been reported.

The present studies were, therefore, undertaken to examine more specifically both intratypic and intertypic specificity between HSV-1 strains (KOS, 17, HFEM, and mP) and HSV-2 strains (186, G, and GP6) in the T cell-mediated immune response and the DTH response to HSV.

MATERIALS AND METHODS

Cells and cell culture. Monolayer cultures of the human cell line HEp-2 were used for the preparation of virus stocks. A continuous line of African green monkey kidney cells (Vero) was used for virus plaque assays. Both HEp-2 and Vero cells were grown at 37°C in Dulbecco modified Eagle medium supplemented

with 10% fetal bovine serum and containing 0.075 or 0.225% $NaHCO_3$ for cultures in closed or open vessels, respectively. C57BL/6 mouse embryo fibroblasts transformed by simian virus 40 (BL/6 WT-3) (43) were used as target cells in the ^{51}Cr release assay.

Viruses and virus assays. HSV-1 strains KOS (42, 45), 17 (4), HFEM (16, 27), and mP (11, 17, 18), and HSV-2 strains 186 (12), G (6, 11, 38), and GP6 (27) have been described. HSV strains KOS, 17, and 186 were kindly provided by Priscilla A. Schaffer; strains mP, G, and GP6 were kindly provided by Bernard Roizman; and strain HFEM was obtained from A. Buchan through Patricia Spear.

Virus stocks were prepared in HEp-2 cells, and titers were determined in Vero cells by a plaque assay utilizing a 2% methylcellulose overlay. All virus stocks were stored at −70°C. The kinetics of virus replication was determined in HEp-2 and BL/6 WT-3 cells. Two-day-old cell monolayers (2×10^5 cells) were infected at a multiplicity of infection (MOI) of 5 and incubated at 37°C for 1 h. The cultures were washed two times with Tris-buffered saline, and 2 ml of medium containing 5% fetal bovine serum was added. The cultures were incubated at 37°C in a 5% CO_2 atmosphere. At designated times, duplicate cultures were frozen and thawed three times and pooled. The resulting suspension was centrifuged at 250 × *g* for 10 min at 4°C, and supernatant fluids representing total virus yields were assayed on Vero cell monolayers at 37°C.

Generation of CTL. Cytotoxic T lymphocytes (CTL) capable of specifically killing syngeneic HSV-infected cells were generated by immunizing C57BL/6 ($H\text{-}2^b$) mice (Jackson Laboratories, Bar Harbor, Maine) in hind footpads with 10^5 PFU of HSV per footpad. The draining lymph nodes were excised 5 days postimmunization, and lymphocyte suspensions were prepared by gently pressing the lymph nodes through a 60-gauge stainless steel wire mesh. Viable cells were counted by trypan blue exclusion and suspended at 4×10^6 lymphocytes per ml in RPMI 1640 media containing 2×10^{-5} M 2-mercaptoethanol, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 100 U of penicillin per ml, 100 g of streptomycin per ml, 0.03% glutamine, 0.225% $NaHCO_3$, and 10% heat-inactivated (56°C, 30 min) fetal bovine serum. Lymphocytes (2×10^7) were added to 60-mm tissue culture dishes and incubated at 37°C in 5% CO_2 for 3 days. For controls, lymphocytes from unimmunized mice were similarly prepared and cultured.

^{51}Cr release assay. The assay was performed as described (5). Briefly, confluent monolayers of target cells growing in 75-cm^2 tissue culture flasks were infected with HSV-1 or HSV-2 at an MOI of 2.5, and 200 μCi of ^{51}Cr was added to each flask. The cells were placed at 37°C for 14 to 16 h. Cells (2×10^4) in 0.1 ml were added to glass culture tubes (10 by 75 mm) with an equal volume of effector lymphocytes.

To determine the ability of HSV-infected, unlabeled cells to compete in a cold cell competition assay, the HSV-infected or mock-infected, unlabeled cells were added to the tubes (10 by 75 mm) at the same time as the ^{51}Cr-labeled target cells at 4°C. Ratios of 5 to 1 and 10 to 1 competing cell to ^{51}Cr-labeled target cell were used. The effector lymphocytes were then added, and the assay was incubated at 37°C for 5 h.

Antibody-dependent complement-mediated cytolysis of HSV-infected cells. Antibody-dependent complement-mediated cytolysis was measured as described previously (5). The serum was prepared by immunization of rabbits with primary rabbit kidney cells infected with either HSV-1 KOS or HSV-2 186.

Adoptive transfer of lymph node cells and measurement of DTH. DTH responses were determined as previously described by Nash and colleagues (28). Briefly, BALB/c mice ($H\text{-}2^d$) (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used at 4 to 8 weeks of age. Donor mice were immunized with 10^5 PFU of virus by hind footpad inoculations. Five to eight days later the draining popliteal lymph nodes were aseptically removed. The lymph node cells (2.0×10^7 to 2.5×10^7 cells per 0.3 ml) were injected intravenously into the tail vein of normal, age-matched, syngeneic recipients. At 1 to 24 h, the mice were anesthetized and challenged with 1.0×10^4 to 2.0×10^4 PFU/50 μl of virus in the pinna of the ear. Ear thickness was then measured at 24 and 48 h post ear challenge with a Mitutoyo dial thickness gauge.

RESULTS

Intratypic specificity of CTL generated with HSV-1 strains KOS, 17, HFEM, and mP. CTL were generated in C57BL/6 mice immunized with HSV-1 strains KOS, 17, HFEM, and mP, as described above. However, initial results with strain HFEM indicated that the lymphocytes generated with 10^5 PFU of HFEM per hind footpad were not cytotoxic to the same degree as those induced by 10^5 PFU of other HSV-1 strains. Attempts were then made to generate CTL with 10^5, 10^6, and 10^7 PFU of HFEM per hind footpad. Lymphocytes generated against KOS with 10^5 PFU were included as positive controls. As shown in Table 1, 10^7 PFU of HFEM per hind footpad induced CTL of a comparable reactivity as the KOS-induced CTL. In this experiment the effector-to-target-cell ratios of 40 to 1 and 20 to 1 were not tested against uninfected target cells due to the insufficient number of CTL generated after in vitro cultivation. However, in other experiments (Tables 2 through 4), the percent specific ^{51}Cr release from uninfected target cells in the presence of CTL generated to either HSV-1 or HSV-2 ranged from 0 to 4.0. All further experiments with HFEM utilized inoculation of 10^7 PFU per hind footpad. The data in Table 1 also demonstrate that both HFEM and KOS-induced lymphocytes are capable of lysing either HFEM- or KOS-infected, ^{51}Cr-labeled target cells.

The CTL generated in response to the four HSV-1 strains were then reacted against ^{51}Cr-labeled target cells infected with KOS, 17, HFEM, or mP. Five independent experiments were carried out, and the results of one representative experiment are presented in Fig. 1. Effector-to-target-cell ratios were varied from 40 to 1 to 5 to 1. The results demonstrate that significant levels of cross-reacting lymphocytes

TABLE 1. Induction of CTL by HSV-1 strains KOS and HFEM

Lymphocyte donor (C57BL/6) immunized with:	Dose (PFU)	Effector cell/target cell ratio	% Specific ^{51}Cr release from BL/6 WT-3 cells infected with [a]:		
			HSV-1 KOS	HSV-1 HFEM	None
HSV-1 KOS	1×10^5	40:1	49.0	31.9	NT[b]
		20:1	32.9	17.9	NT
		10:1	27.3	9.7	0.6
HSV-1 HFEM	1×10^5	40:1	NT	NT	NT
		20:1	7.2	5.3	NT
		10:1	5.3	4.0	1.4
HSV-1 HFEM	1×10^6	40:1	31.6	21.6	NT
		20:1	23.0	11.1	NT
		10:1	13.4	8.2	2.1
HSV-1 HFEM	1×10^7	40:1	46.3	27.4	NT
		20:1	42.8	25.8	NT
		10:1	26.6	17.9	1.5

[a] Lymphocyte cytotoxicity was determined in a 5-h ^{51}Cr release assay at 37°C. Target cells were either infected with HSV-1 strains KOS or HFEM at an MOI of 2.5 or mock-infected, and cells were harvested at 14 h postinfection.

[b] NT, Not tested. The 40:1 effector-cell-to-target-cell ratio could not be performed with HFEM effector cells generated by an immunizing dose of 1×10^5 PFU due to insufficient numbers of these effectors.

are induced by all HSV-1 strains examined and that HSV-1-infected target cells are all equally susceptible to lysis by HSV-1-induced CTL.

Intratypic specificity of CTL generated with HSV-2 strains 186, G, and GP6 and intertypic specificity with HSV-1 KOS CTL. Similar experiments were performed with HSV-2 strains 186, G, and GP6. Initial experiments with HSV-2 strains yielded low but significant values of percent specific ^{51}Cr release when HSV-2-in-

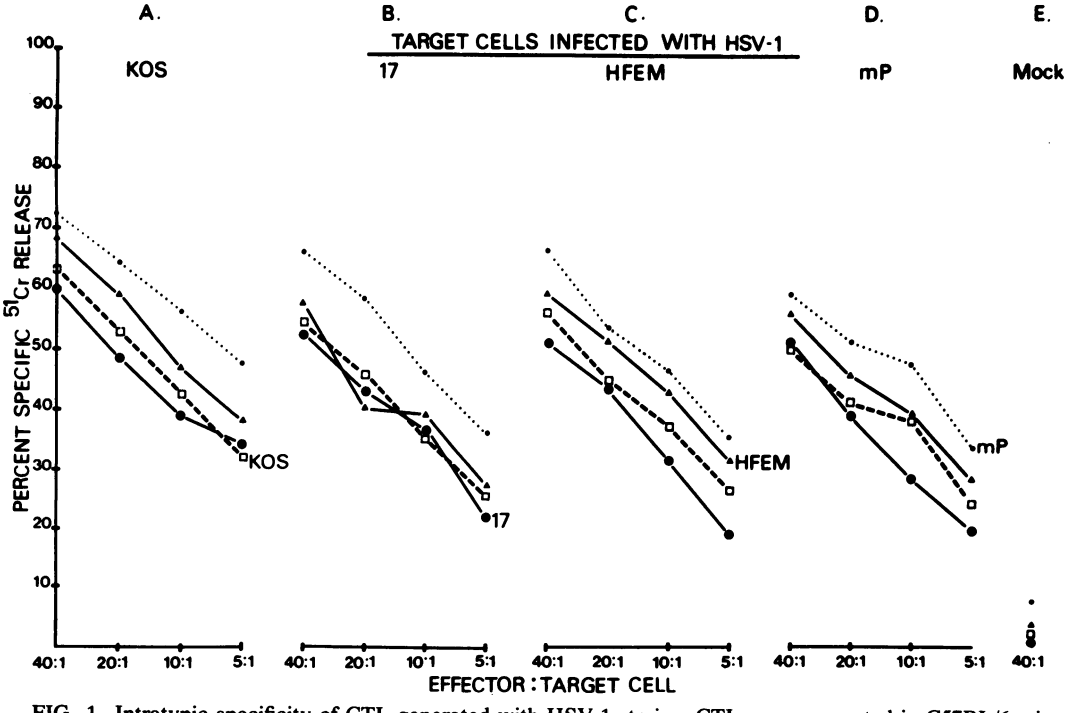

FIG. 1. Intratypic specificity of CTL generated with HSV-1 strains. CTL were generated in C57BL/6 mice with HSV-1 strains KOS, 17, HFEM, and mP and used in the standard ^{51}Cr release assay. Effector-to-target-cell ratios were varied from 40 to 1 to 5 to 1. The HSV-1 CTL populations were reacted against BL/6 WT-3 target cells infected with KOS (A), 17 (B), HFEM (C), mP (D), and mock (E). Target cells were infected at an MOI of 2.5, labeled with 200 μCi of ^{51}Cr and harvested at 14 h postinfection. In each panel the homologous CTL is labeled: KOS (□--□), 17 (●--●), HFEM (▲--▲), mP (●···●).

TABLE 2. Intratypic specificity between HSV-2 strains 186, G, and GP6 and intertypic specificity between HSV-1 KOS and HSV-2 strains

Lymphocyte donor (C57BL/6) immunized with:	Effector cell/target cell ratio	% Specific ^{51}Cr release from BL/6 WT-3 cells infected with [a]:				
		HSV-2 186	HSV-2 G	HSV-2 GP6	HSV-1 KOS	None
HSV-2 186	40:1	11.1	12.1	19.7	42.9	3.2
	20:1	7.7	6.3	9.1	32.5	NT[b]
HSV-2 G	40:1	14.0	12.3	17.8	44.2	2.7
	20:1	11.1	6.7	11.4	34.7	NT
HSV-2 GP6	40:1	10.5	6.4	24.6	31.9	0.0
	20:1	6.3	4.3	10.3	19.6	NT
HSV-1 KOS	40:1	10.2	3.6	8.2	49.3	1.3
	20:1	9.5	3.3	6.7	37.7	NT

[a] Lymphocyte cytotoxicity was determined in a 5-h ^{51}Cr release assay at 37°C. Cells were either infected with HSV-2 strains 186, G, or GP6, HSV-1 strain KOS at an MOI of 2.5, or mock-infected, and cells were harvested at 14 h postinfection.

[b] NT, Not tested.

fected target cells were used. However, when HSV-2 CTL were reacted against HSV-1-infected targets, two- to fourfold more release of ^{51}Cr was obtained (Table 2). These results are reproducible, and this same low susceptibility of HSV-2-infected target cells has also been observed in BALB/c, C3H, and CBA/J mice (data not shown). The results in Table 2 demonstrate that all HSV-2 strains induce CTL which are cross-reactive and that these CTL are more cytotoxic for HSV-1-infected target cells than for the HSV-2-infected target cells.

Growth of HSV in WT-3 cells. The lowered susceptibility of HSV-2-infected target cells to lysis by either HSV-1 or HSV-2 CTL could be the result of inefficient replication in BL/6 WT-3 cells. A growth curve experiment was performed using HSV-1 strains KOS, 17, and HFEM and HSV-2 strains 186, G, and GP6 in WT-3 cells. Although HSV-1 KOS and HFEM showed more efficient replication in WT-3 cells, HSV-1 17 replicated with approximately the same efficiency as HSV-2 186, G, and GP6 (Fig. 2). Similar results were obtained when the same strains were grown in the human cell line HEp-2 (data not shown). Infectious center assays were also performed to determine whether similar numbers of cells were infected with HSV-1 and -2. The results indicate that there is no difference between the number of cells infected by either HSV-1 KOS or HSV-2 186 which are capable of forming an infectious center on Vero cells (data not shown).

Effect of increasing the MOI of target cells on their susceptibility to lysis by CTL. In an attempt to increase the susceptibility of HSV-2-infected targets to lysis by HSV-induced CTL, C57BL/6 WT-3 cells were infected at various MOI from 1 to 20, and the cells were then harvested at 14 h postinfection and used in the ^{51}Cr release assay. Increasing the MOI of either HSV-1- or HSV-2-infected target cells did not increase the percent specific ^{51}Cr release obtained (Table 3).

Time course of susceptibility of HSV-1- and HSV-2-infected BL/6 WT-3 cells to lysis by HSV-1 and HSV-2 CTL. The replicative cycles of HSV-1 and HSV-2 have been shown to differ (13). We therefore examined the susceptibility of HSV-1- and HSV-2-infected target cells between 2 and 16 h postinfection to determine whether there was an optimal time for the expression of antigens that react with CTL in the ^{51}Cr release assay. Figure 3 shows the results of one of three independent experiments in which HSV-1 and HSV-2 CTL were reacted against both HSV-1- and HSV-2-infected target cells. Between 2 and 16 h postinfection HSV-1-infected targets were susceptible to lysis by both HSV-1 and HSV-2 CTL. The level of susceptibility of HSV-1-infected target cells increased between 2 and 12 h postinfection. However, HSV-2-infected target cells were not highly susceptible to lysis by either HSV-1 or HSV-2 CTL. A slight increase was observed in the lysis of HSV-2-infected target cells between 2 and 16 h postinfection, but the level of susceptibility never approached the level of HSV-1-infected target cells.

Effect of increasing the effector-to-target-cell ratio on lysis of HSV-2-infected target cells. Another approach to increase the level of susceptibility of HSV-2-infected target cells was to increase the effector-to-target-cell ratio. The results in Fig. 4 illustrate that increasing the effector-to-target-cell ratio from 25 to 1 to 100 to 1 did cause an increase in the percent specific ^{51}Cr release from both HSV-1- and HSV-2-infected target cells. At the 100-to-1 ratio HSV-2-infected target cells were lysed by HSV-1 and HSV-2 CTL to values of 31 and 36% specific ^{51}Cr release, respectively. However, at the same ratio the HSV-1-infected target cells were lysed to values of 78 and 83% percent specific

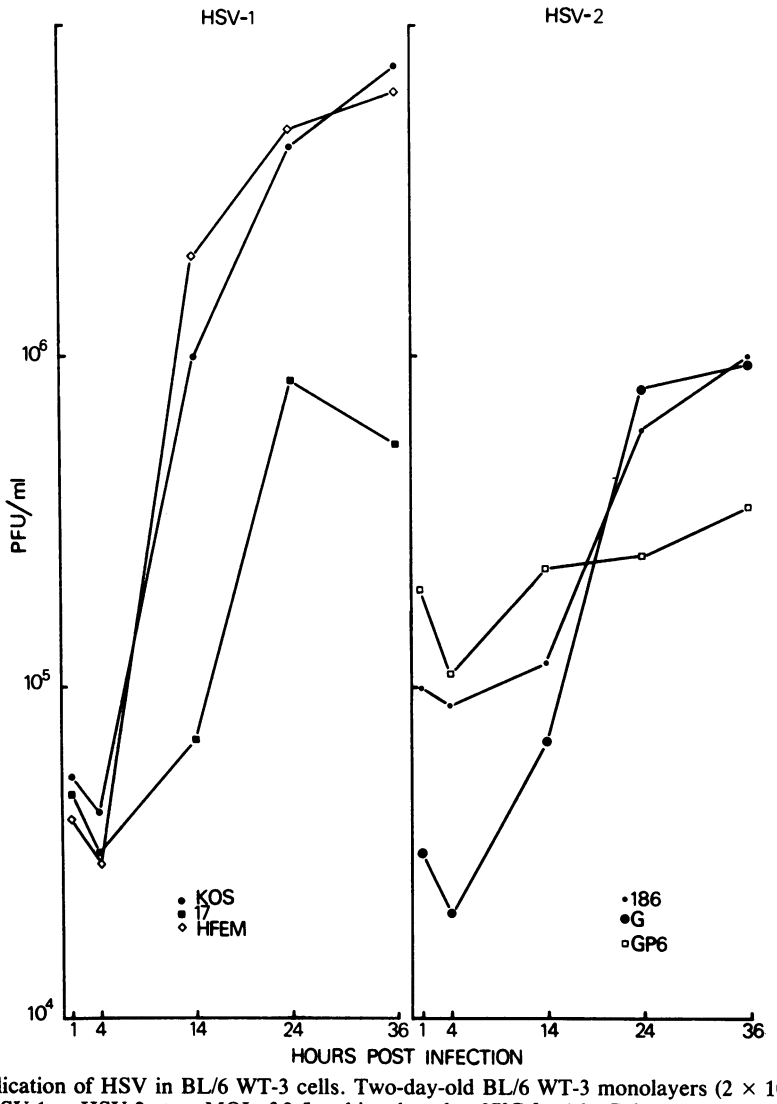

FIG. 2. Replication of HSV in BL/6 WT-3 cells. Two-day-old BL/6 WT-3 monolayers (2×10^5 cells) were infected with HSV-1 or HSV-2 at an MOI of 2.5 and incubated at 37°C for 1 h. Cultures were then washed two times with Tris-buffered saline, and 2 ml of Dulbecco medium containing 5% fetal bovine serum was added. Cultures were incubated in a WEDCO CO_2 incubator at 37°C. At the designated time point, duplicate cultures were harvested by freezing and thawing three times, and cellular debris was removed by centrifugation at $250 \times g$ for 10 min. Supernatant fluids representing total virus yields were assayed on Vero cell monolayers.

^{51}Cr release by HSV-2 and HSV-1 CTL. Therefore, the HSV-2-infected target cells were susceptible to lysis by CTL, but the level of killing was two- to threefold less than that of the HSV-1-infected target cells.

Ability of unlabeled HSV-1- and HSV-2-infected BL/6 WT-3 cells to compete for CTL. The cold cell competition assay yields information on the recognition of target cells by CTL. Various numbers of infected, unlabeled cells were mixed with infected ^{51}Cr-labeled target cells, and the CTL were then added to this mixture. The ability of the unlabeled "cold" HSV-2-infected cells to compete with the ^{51}Cr-labeled target cell was measured as a reduction in the percent specific ^{51}Cr release. The results of such an experiment utilizing both HSV-1- and HSV-2-infected cold cells are shown in Fig. 5 with both HSV-1 and HSV-2 CTL. Figure 5A shows the results of HSV-1 and HSV-2 cold cells competing for the HSV-1 CTL against HSV-1-infected ^{51}Cr-labeled target cells. At both the 5 to 1 and 10 to 1 inhibitor-cell-to-target-cell ratio, both HSV-1 and HSV-2 cold cells were capable of reducing the value of percent specific ^{51}Cr release of the control by approximately 50%.

TABLE 3. Effect of various MOIs on the susceptibility of HSV-infected target cells to lysis by HSV CTL

Target cells (Bl/6 WT-3) infected by[a]:	MOI	% Specific ^{51}Cr release by CTL generated against[b]:	
		HSV-1 KOS	HSV-2 186
HSV-1 KOS	1	39.7	27.8
	2.5	42.6	38.1
	5	35.2	29.0
	10	41.0	31.5
	20	35.8	30.5
HSV-2 186	1	5.8	8.0
	2.5	2.1	6.1
	5	1.6	4.9
	10	6.6	7.3
	20	7.5	10.4
None		3.1	4.9

[a] BL/6 WT-3 target cells were either infected with HSV-1 KOS, HSV-2 186, or mock-infected, and cells were harvested at 14 h postinfection.

[b] Lymphocyte cytotoxicity was determined in a 5-h ^{51}Cr release assay at an effector-to-target-cell ratio of 40 to 1. Lymphocyte donors (C57BL/6) were immunized with either HSV-1 KOS or HSV-2 186.

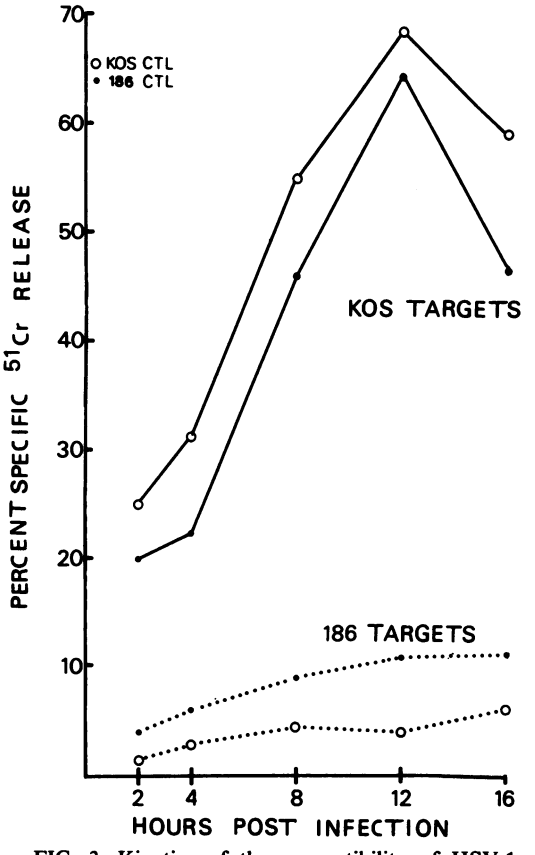

FIG. 3. Kinetics of the susceptibility of HSV-1- and HSV-2-infected BL/6 WT-3 cells to lysis by HSV-1 and HSV-2 CTL. BL/6 WT-3 cells were infected with HSV-1 KOS or HSV-2 186 at an MOI of 2.5 and harvested at various times postinfection. The ^{51}Cr-labeled, HSV-infected target cells were used in the standard ^{51}Cr release assay at an effector-to-target-cell ratio of 40 to 1.

Figure 5B shows the results of the same experiment with HSV-2 CTL. HSV-2 cold cells were capable of reducing the control value by approximately 60%. Since no difference was observed in the ability of HSV-1- and HSV-2-infected cold cells to compete for CTL, both HSV-1- and HSV-2-infected cells must be recognized equally by the CTL. These results indicate that recognition of HSV-2-infected cells does occur even though they are not lysed as efficiently as HSV-1-infected cells. The assay system used, however, does not rigorously rule out the possibility of the existence of type-specific CTL.

Lysis of HSV-1- and HSV-2-infected BL/6 WT-3 cells by antibody-dependent complement-mediated lysis. HSV-1- and HSV-2-infected WT-3 cells were tested for their susceptibility to lysis by antibody and complement. The lysis of HSV-infected cells by antibody and complement has previously been shown to involve the HSV-specific glycoproteins (5, 35). HSV-1- and HSV-2-infected WT-3 cells should, therefore, be lysed by HSV-specific antisera and complement if the virus-specific glycoproteins are expressed on the infected cell surface. HSV-1- and HSV-2-immune sera from rabbits was heat-inactivated for 30 min at 56°C before use in the assay. The results in Table 4 show that WT-3 cells infected with HSV-2 186 were highly susceptible to lysis by antibody and complement, yielding values of 32 and 52% and 67 and 27% specific ^{51}Cr release in the presence of antisera generated against HSV-1 KOS and HSV-2 186, respectively. The HSV-2-infected target cells were, therefore, as susceptible as HSV-1-infected target cells to lysis by HSV-immune serum and complement.

Intertypic specificity of HSV-1- and HSV-2-induced lymphocytes in a DTH assay. The DTH response to HSV was also used to examine cross-reactivity between HSV-1 KOS and HSV-2 186. Lymph node cells were prepared as described above. Ear swelling was measured for lymphocytes transferred on days 6, 7, and 8 after immunization of mice to determine the optimal time of transfer. Lymphocytes transferred on day 7 showed optimal reactivity 48 h after challenge with HSV-1 KOS in the pinna of the ear (data not shown). The specificity of the DTH response was then examined with lymphocytes transferred on day 7 post-immunization and HSV-1 KOS and simian virus 40 to challenge. Only mice receiving HSV-1 KOS-immune lymphocytes and a challenge of HSV-1 KOS responded with an increase in ear thickness (data

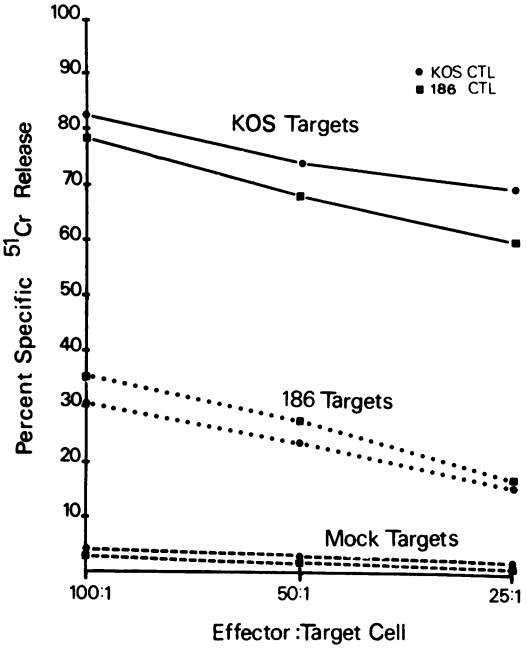

FIG. 4. The effect of increasing the effector-to-target-cell ratio on the lysis of HSV-infected cells. CTL were generated in C57BL/6 mice with HSV-1 KOS and HSV-2 186. BL/6 WT-3 cells were infected at an MOI of 2.5 and harvested at 14 h postinfection for use in the standard ^{51}Cr release assay. Effector-cell-to-target-cell ratios were varied from 100 to 1 to 25 to 1.

not shown). Mice who received HSV-1 KOS-immune lymphocytes did not respond to a challenge of simian virus 40 in the pinna of the ear. Similarly, mice who received normal lymphocytes did not respond to either HSV-1 KOS or simian virus 40. These results confirm previously published findings on the DTH response to HSV (28).

The cross-reactivity of HSV-1 KOS and HSV-2 186 in generating a DTH response was next examined with lymphocytes harvested at 7 days post-immunization (Table 5). Lymphocytes generated against HSV-1 KOS were capable of eliciting a DTH response when mice were challenged with either HSV-1 KOS or HSV-2 186 (Table 5, experiments 1 and 2), and HSV-2 186-immune lymphocytes were capable of eliciting a DTH response against either HSV-1 KOS or HSV-2 186 (Table 5, experiments 3 and 4). The DTH response was measured at 24 and 48 h postchallenge. Maximal responses were observed at 48 h postchallenge, and therefore only the data from 48 h are reported in Table 5. The measurements for mice receiving HSV-immune lymph node cells were significant in all cases as compared with animals who did not receive lymph node cells. A statistical analysis of the difference between mice adoptively transferred with immune lymph node cells and challenged in the ear with the homologous or heterologous serotype of HSV was also done. In all comparisons, no significant difference was observed ($P \geqq 0.05$). This suggests that the DTH effector cells recognize cross-reactive viral determinants.

DISCUSSION

The results presented in this study demonstrate that high levels of cross-reactive lymphocytes are generated in response to infection of mice with HSV-1 and HSV-2. The effector cell populations induced in these studies contain lymphocytes which exhibit both intertypic and intratypic cross-reactivity between HSV-1 (strains KOS, 17, HFEM, and mP) and HSV-2 (strains 186, G, and GP6). Two cell-mediated immune reactions were used to examine the generation of HSV-specific lymphocytes. The in vitro lysis of virus-infected cells by CTL is dependent on CTL recognition of both viral antigens and products of the H-2K or H-2D loci or both of the major histocompatibility complex (8). Previous work from our laboratory (5) and others (23, 24) has demonstrated that the CTL response to HSV infection involves the virus-specific glycoproteins both in the induction of CTL and as target antigens on the surface of virus-infected cells.

The induction of both type-specific and cross-reactive CTL to HSV has been reported (39, 40, 50). The experimental approach yielding a type-specific CTL response was identical to the induction procedure used in this study. However, Pfizenmaier and co-workers used HSV-1 strain Lennette and HSV-2 strain D316 to induce CTL, and HSV-infected peritoneal macrophages were used as syngeneic target cells (39, 40). Another approach yielding cross-reactive CTL used HSV-1 strain Thea and HSV-2 strain Haase to generate CTL from the spleen and peritoneal cavity of the mouse (50). Additional studies are therefore needed to determine the role of the HSV strain, the route of inoculation of virus, and the target cell in the induction of either type-specific or cross-reactive populations of CTL.

The in vivo assay of DTH involves recognition of viral antigens, but the effector cell population is restricted to the Ia subregion of the major histocompatibility complex (32). Although the DTH response can be transferred by Ia compatible effector cells, the rapid clearance of virus also necessitates H-2K and H-2D region compatibility (32). HSV-1-sensitized lymph node cells can be stimulated by either HSV-1 or HSV-2 as measured by [^{3}H]thymidine incorporation (33), but the level of incorporation of

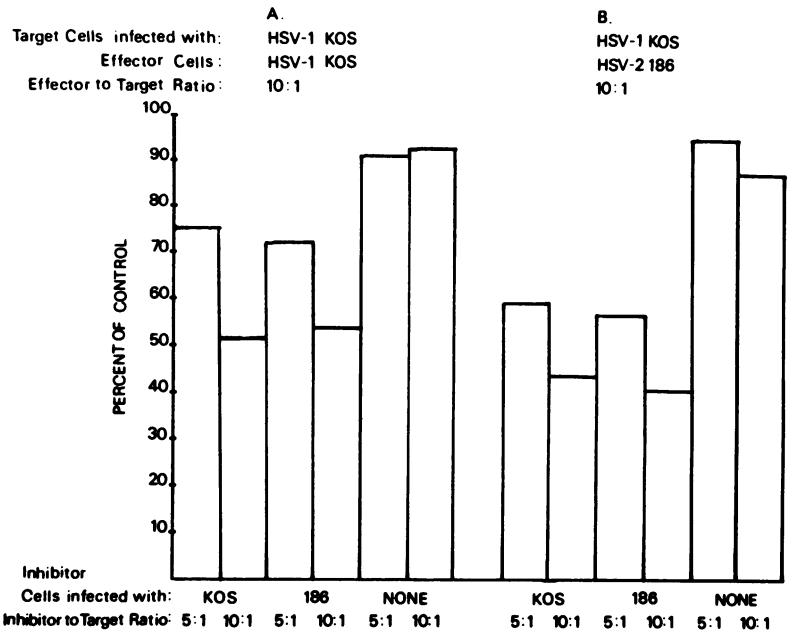

FIG. 5. The effect of HSV-1 and HSV-2-infected cells in the cold cell competition assay. CTL were generated in C57BL/6 mice with HSV-1 KOS and HSV-2 186 and used at an effector-to-target-cell ratio of 10 to 1. BL/6 WT-3 cells were infected at an MOI of 2.5 or mock-infected, and half of the cultures were labeled with 200 μCi of ^{51}Cr. All cells were harvested at 14 h postinfection and ^{51}Cr labeled; HSV-1-, HSV-2-, or mock-infected cells were mixed at 4°C with unlabeled HSV-1-, HSV-2-, or mock-infected cells at ratios of 5 to 1 and 10 to 1, unlabeled to labeled cells. CTL were then added to the cell mixture, and a 5-h ^{51}Cr release assay was carried out. Cultures were also run without unlabeled cells to serve as positive controls.

[^{3}H]thymidine is fivefold-less when HSV-1-sensitized lymph node cells are stimulated by HSV-2. A suppression of the DTH response to HSV has also been reported, and the HSV-specific DTH response is suppressed by either HSV-1 or HSV-2 (30, 31). The decreased level of suppression induced by the heterologous virus indicates that the DTH effector cell population contains cells which recognize some type-common antigenic determinants and some type-specific antigenic determinants of HSV-1 and HSV-2 (30). Similar results have been obtained in the influenza virus system. The DTH response and the CTL response are restricted to similar regions of the H-2 complex (8, 25), and significant levels of cross-reacting lymphocytes are detected (1, 3, 46, 57). Studies of reovirus indicate that CTL exhibit serotype specificity which segregates with the S1 genome segment, but cross-reactive CTL are found when identity at the S1 genome segment is not observed (14). However, the antigenic determinants involved in the generation of reovirus cross-reactive CTL were not identified.

Antigenic studies of the HSV-specific glycoproteins indicate that both type-common and type-specific antigenic sites exist on the glycoprotein molecules (7, 10, 55). HSV-1 specifies the synthesis of at least four antigenically distinct glycoproteins (2, 54), designated gB, gC, gD, and gE. A fifth glycoprotein, gA, has recently been shown to share antigenic determinants with gB (9, 37), and a precursor to product (gA to gB) relationship has been proposed (9). All of the virus-specific glycoproteins have been physically mapped using HSV-1 × HSV-2 intertypic recombinants (49), and only gG of HSV-1 and HSV-2 do not map to identical locations on the genomes. Additionally, only type-specific antigenic determinants have been identified on gC (55). The involvement of HSV-specific glycoproteins in the CTL response and the induction of high levels of cross-reacting CTL reported in this study suggest that glycoproteins bearing type-common antigenic sites (gA/gB, gD) function in the induction of cross-reactive HSV CTL. The effector lymphocyte population may also contain type-specific CTL, but these cells are not detected by our assay system.

An apparent lowered susceptibility to lysis by CTL of HSV-2-infected target cells was observed during these studies. HSV-2-infected cells were consistently found to be two- to threefold-less susceptible to lysis in the CTL

TABLE 4. Comparative susceptibility of HSV-1- and HSV-2-infected BL/6 WT-3 cells to antibody-dependent complement-mediated lysis

Target cells (BL/6 WT-3) infected by:	HSV-immune serum[a]	Complement[b]	% Specific ^{51}Cr release[c]	
			Expt 1	Expt 2
HSV-1 KOS	α KOS	+	67	46
	α 186	+	53	49
HSV-2 186	α KOS	+	32	52
	α 186	+	67	27
None	α KOS	+	3.5	8.2
	α 186	+	0.1	4.6

[a] Antiserum to HSV-1 KOS and HSV-2 186 was generated in rabbits and used at a 1:10 dilution after heat inactivation at 56°C for 30 min.

[b] Guinea pig complement was used at a final dilution of 1:8. Complement was also heat-inactivated at 56°C for 30 min and used in duplicate tubes as a negative control. The percent specific ^{51}Cr release in the presence of antiserum and heat-inactivated complement was not greater than the values obtained with mock-infected BL/6 WT-3 cells.

[c] Cytotoxic activity was determined in a 5-h ^{51}Cr release assay at 37°C. Cells were either infected with HSV-1 KOS, HSV-2 186 (MOI of 2.5) or mock-infected, and cells were harvested at 14 h postinfection.

assay with either HSV-1 or HSV-2-induced CTL. Efforts were focused on determining the reason for this altered susceptibility, and the results suggested that the lowered susceptibility of HSV-2-infected target cells was not due to a lack of viral glycoproteins on the cell surface as assayed by antibody and complement-mediated cytolysis. Assays of growth of HSV-1 and HSV-2 in mouse cells and infectious center assays yielded data which suggested that the altered susceptibility could not be attributed to an inability of the virus to grow in the C57BL/6 mouse cells.

Despite the lowered susceptibility of HSV-2-infected target cells to lysis by HSV-1 or HSV-2-induced CTL, highly reactive CTL were generated in C57BL/6 mice by the HSV-2 strains examined in this study. HSV-2-induced CTL were as cytotoxic as HSV-1-induced CTL against HSV-1-infected target cells. We are currently attempting to generate a type-specific CTL response to HSV-1 and -2 to examine the role of specific glycoproteins in the induction of CTL. The function of specific glycoproteins as target antigens on the infected target cell surface will also be examined with HSV-1 × HSV-2 intertypic recombinants.

ACKNOWLEDGMENT

This study was supported by research grants CA 27503, CA 18450, and CA 09124 from the National Cancer Institute, National Institutes of Health, Bethesda, Md.

TABLE 5. Cross-reactivity of HSV-1 and -2 in a DTH assay

Expt	LNC donors immunized with[a]:	Mice challenged with[b]:	Increase in ear thickness[c] (48 h)	Significance[d]
1	HSV-1 KOS	HSV-1 KOS	12.6 ± 2.5	$P = 0.02$
	None	HSV-1 KOS	1.3 ± 1.1	
	HSV-1 KOS	HSV-2 186	9.5 ± 0.7	$P = 0.02$
	None	HSV-2 186	6.3 ± 0.5	
2	HSV-1 KOS	HSV-1 KOS	11 ± 5	$P = 0.02$
	None	HSV-1 KOS	3 ± 2.2	
	HSV-1 KOS	HSV-2 186	8.2 ± 2.6	$P = 0.02$
	None	HSV-2 186	3.6 ± 1.3	
3	HSV-2 186	HSV-2 186	15.1 ± 2.6	$P < 0.001$
	None	HSV-2 186	5.1 ± 1.6	
	HSV-2 186	HSV-1 KOS	13.7 ± 3.3	$P = 0.001$
	None	HSV-1 KOS	3.2 ± 1.3	
4	HSV-2 186	HSV-2 186	14.6 ± 3.5	$P = 0.01$
	None	HSV-2 186	2.7 ± 1.5	
	HSV-2 186	HSV-1 KOS	10.7 ± 2.2	$P < 0.001$
	None	HSV-1 KOS	0.8 ± 1.3	

[a] Lymph node cells (LNC) (2.0×10^7 to 2.5×10^7) obtained from BALB/c mice immunized 5 to 8 days earlier with 10^5 PFU of HSV-1 KOS or HSV-2 186 were injected intravenously into normal, age-matched, syngeneic recipients. Control mice did not receive lymph node cells.

[b] One to twenty-four hours after adoptive transfer of lymph node cells, 1×10^4 to 2×10^4 PFU per 50 μl of a CsCl-gradient-purified preparation of HSV-1 KOS or HSV-2 186 was injected into the ear pinna of all mice.

[c] All mice were anesthetized, and measurements of their ear thickness were taken before challenge with virus in the ear and at 24 and 48 h postchallenge. The data shown are the mean ± 1 standard deviation of the simple arithmetic increase in ear thickness for each group (three to six mice per group).

[d] Student *t*-test was used to determine statistical significance. The *P*-values indicate the difference between mice adoptively transferred with immune lymph node cells and control mice.

LITERATURE CITED

1. **Askonas, B. A., and R. G. Webster.** 1980. Monoclonal antibodies to hemagglutinin and to H-2 inhibit the cross-reactive cytotoxic T cell populations induced by influenza. Eur. J. Immunol. **10**:151–156.
2. **Baucke, R. B., and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex virus. V. Identification of an Fc-binding glycoprotein. J. Virol. **32**:779–789.
3. **Braciale, T. J.** 1979. Specificity of cytotoxic T cells directed to influenza virus hemagglutinin. J. Exp. Med. **149**:856–869.
4. **Brown, S. M., D. A. Ritchie, and J. H. Subak-Sharpe.** 1973. Genetic studies with herpes simplex virus type 1: the isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. J. Gen. Virol. **18**:329–346.
5. **Carter, V. C., P. A. Schaffer, and S. S. Tevethia.** 1981. The involvement of herpes simplex virus type 1 glycoproteins in cell-mediated immunity. J. Immunol. **126**:1655–1660.
6. **Cassai, E., R. Manservigi, A. Corallini, and M. Terni.** 1975/76. Plaque dissociation of herpes simplex viruses: biochemical and biological characters of the viral variants. Intervirology **6**:212–223.
7. **Cohen, G. H., M. Katze, C. Hydrean-Stern, and R. J. Eisenberg.** 1978. Type-common CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular-weight envelope glycoprotein. J. Virol. **27**:172–181.
8. **Doherty, P. C., R. V. Blanden, and R. M. Zinkernagel.** 1976. Specificity of virus-immune effector T cells for H2-K and H2-D compatible interactions: implications for H antigen diversity. Transplant. Rev. **29**:89–124.
9. **Eberle, R., and R. J. Courtney.** 1980. gA and gB glycoproteins of herpes simplex virus type 1: two forms of a single polypeptide. J. Virol. **36**:665–675.
10. **Eisenberg, R. J., M. Ponce de Leon, and G. H. Cohen.** 1980. Comparative structural analysis of glycoprotein gD of herpes simplex virus types 1 and 2. J. Virol. **35**:428–435.
11. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effects on social behavior of infected cells. J. Gen. Virol. **2**:357–364.
12. **Esparza, J., D. J. M. Purifoy, P. A. Schaffer, and M. Benyesh-Melnick.** 1974. Isolation, complementation and preliminary phenotypic characterization of temperature-sensitive mutants of herpes simplex virus type 2. Virology **57**:554–565.
13. **Fenwick, M., L. S. Morse, and B. Roizman.** 1979. Anatomy of herpes simplex virus DNA. XI. Apparent clustering of functions effecting rapid inhibition of host DNA and protein synthesis. J. Virol. **29**:825–827.
14. **Finberg, R., H. L. Weiner, B. N. Fields, B. Benacerraf, and S. J. Burakoff.** 1979. Generation of cytolytic T lymphocytes after reovirus infection: role of the S1 gene. Proc. Natl. Acad. Sci. U.S.A. **76**:442–446.
15. **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 antibodies. J. Gen. Virol. **40**:443–454.
16. **Halliburton, I. W., R. E. Randall, R. A. Killington, and D. H. Watson.** 1977. Some properties of intertypic recombinants between type 1 and type 2 herpes simplex virus. J. Gen. Virol. **36**:471–484.
17. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. **14**:640–651.
18. **Hoggan, M. D., and B. Roizman.** 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. **70**:208–219.
19. **Honess, R. W., and D. H. Watson.** 1974. Herpes simplex virus-specific polypeptides studied by polyacrylamide gel electrophoresis of immune precipitates. J. Gen. Virol. **22**:171–185.
20. **Howes, E. L., W. Taylor, N. A. Mitchison, and E. Simpson.** 1979. MHC matching shows that at least two T-cell subsets determine resistance to HSV. Nature (London) **277**:67–68.
21. **Kieff, E. D., B. Hoyer, S. L. Bachenheimer, and B. Roizman.** 1972. Genetic relatedness of type 1 and type 2 herpes simplex viruses. J. Virol. **9**:738–745.
22. **Lausch, R., J. Swyers, and H. Kaufman.** 1966. Delayed hypersensitivity to herpes simplex virus in the guinea pig. J. Immunol. **96**:981–987.
23. **Lawman, M. J. P., P. T. Naylor, L. Huang, R. J. Courtney, and B. T. Rouse.** 1981. Cell-mediated immunity to herpes simplex virus: induction of cytotoxic T lymphocyte responses by viral antigens incorporated into liposomes. J. Immunol. **126**:304–308.
24. **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.
25. **Leung, K.-N., G. L. Ada, and I. F. C. McKenzie.** 1980. Specificity, Ly phenotype and H-2 compatibility requirements of effector cells in delayed-type hypersensitivity responses to murine influenza virus infection. J. Exp. Med. **151**:815–826.
26. **Morse, L. S., T. G. Buchman, B. Roizman, and P. A. Schaffer.** 1977. Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA arrangements in the generation of intertypic (HSV-1 × HSV-2) recombinants. J. Virol. **24**:231–248.
27. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 × HSV-2 recombinants. J. Virol. **26**:389–410.
28. **Nash, A. A., H. J. Field, and R. Quartey-Papafio.** 1980. Cell-mediated immunity in herpes simplex virus-infected mice: induction, characterization and antiviral effects of delayed type hypersensitivity. J. Gen. Virol. **48**:351–357.
29. **Nash, A. A., and P. G. H. Gell.** 1980. Cell-mediated immunity in herpes simplex virus-infected mice: suppression of delayed hypersensitivity by an antigen-specific B lymphocyte. J. Gen. Virol. **48**:359–364.
30. **Nash, A. A., P. G. H. Gell, and P. Wildy.** 1981. Tolerance and immunity in mice infected with herpes simplex virus: simultaneous induction of protective immunity and tolerance to delayed-type hypersensitivity. Immunology **43**:153–159.
31. **Nash, A. A., J. Phelan, P. G. H. Gell, and P. Wildy.** 1981. Tolerance and immunity in mice infected with herpes simplex virus: studies on the mechanism of tolerance to delayed-type hypersensitivity. Immunology **43**:363–369.
32. **Nash, A. A., J. Phelan, and P. Wildy.** 1981. Cell-mediated immunity in herpes simplex virus-infected mice: H-2 mapping of the delayed-type hypersensitivity response and the antiviral T cell response. J. Immunol. **126**:1260–1262.
33. **Nash, A. A., R. Quartey-Papafio, and P. Wildy.** 1980. Cell-mediated immunity in herpes simplex virus-infected mice: functional analysis of lymph node cells during periods of acute and latent infection, with reference to cytotoxic and memory cells. J. Gen. Virol. **49**:309–317.
34. **Norrild, B., S. L. Shore, T. L. Cromeans, and A. J. Nahmias.** 1980. Participation of three major glycoprotein antigens of herpes simplex virus type 1 early in the infectious cycle as determined by antibody-dependent cell-mediated cytotoxicity. Infect. Immun. **28**:38–44.
35. **Norrild, B., S. L. Shore, and A. J. Nahmias.** 1979. Herpes simplex virus glycoproteins: participation of individual herpes simplex virus type 1 glycoprotein antigens in immunocytolysis and their correlation with previously identified glycopeptides. J. Virol. **32**:741–748.
36. **Para, M. F., R. B. Baucke, and P. G. Spear.** 1980.

Immunoglobulin G (Fc)-binding receptors on virions of herpes simplex virus type 1 and transfer of these receptors to the cell surface by infection. J. Virol. **34**:512–520.

37. **Pereira, L., D. Dondero, B. Norrild, and B. Roizman.** 1981. Differential reactivity and processing of glycoproteins gA and gB of herpes simplex virus types 1 and 2 made in Vero and HEp-2 cells. Proc. Natl. Acad. Sci. U.S.A. **78**:5202–5206.
38. **Periera, L., M. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus synthesis. V. Properties of α polypeptides specified by HSV-1 and HSV-2. Virology **77**:733–749.
39. **Pfizenmaier, K., H. Jung, A. Starzinski-Powitz, M. Röllinghoff, and H. Wagner.** 1977. The role of T cells in anti-herpes simplex virus. I. Induction of antigen-specific cytotoxic T lymphocytes. J. Immunol. **119**:939–944.
40. **Pfizenmaier, K., H. Jung, A. Starzinski-Powitz, M. Röllinghoff, and H. Wagner.** 1977. T cell mediated cytotoxicity against herpes simplex virus-infected target cells. Nature (London) **265**:630–632.
41. **Plummer, G.** 1973. A review of the identification and titration of antibodies to herpes simplex virus type 1 and type 2 in human sera. Cancer Res. **33**:1469–1476.
42. **Powell, K., and R. J. Courtney.** 1975. Polypeptides synthesized in herpes simplex virus type 2 infected HEp-2 cells. Virology **66**:217–228.
43. **Pretell, J., R. S. Greenfield, and S. S. Tevethia.** 1979. Biology of simian virus 40 (SV40) transplantation rejection antigen (TrAg). V. *In vitro* demonstration of SV40 TrAg in SV40 infected nonpermissive mouse cells by the lymphocyte mediated cytotoxicity assay. Virology **97**:32–41.
44. **Rager-Zisman, B., and A. C. Allison.** 1976. Mechanism of immunologic resistance to herpes simplex virus 1 (HSV-1) infection. J. Immunol. **116**:35–40.
45. **Rawls, N. E., D. Laurel, J. L. Melnick, J. H. Glickman, and R. H. Kaufman.** 1968. A search for viruses in smegma, premalignant and early malignant cervical tissues. The isolation of herpesviruses with distinct antigenic properties. Am. J. Epidemiol. **87**:647–655.
46. **Reiss, C. S., and J. L. Schulman.** 1980. Influenza type A virus M protein expression on infected cells is responsible for cross-reactive recognition by cytotoxic thymus-derived lymphocytes. Infect. Immun. **29**:719–723.
47. **Rogers, H. W., L. V. Scott, and R. A. Patnode.** 1972. Sensitization of guinea pigs to herpes simplex virus. J. Immunol. **109**:801–806.
48. **Roizman, B.** 1979. The structure and isomerization of herpes simplex virus genomes. Cell **16**:481–494.
49. **Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29**:677–697.
50. **Sethi, K. K., and H. Brandis.** 1977. Specifically immune mouse T cells can destroy H-2 compatible murine target cells infected with herpes simplex virus types 1 and 2. Z. Immunitaeforsch. **150**:162–173.
51. **Sethi, K. K., and M. H. Wolff.** 1980. The nature of host-cell herpes-simplex virus interaction(s) that render cells susceptible to virus-specific cytotoxic T cells. Immunobiology. **157**:365–378.
52. **Sim, C., and D. H. Watson.** 1973. The role of type specific and cross reacting structural antigens in the neutralization of herpes simplex virus types 1 and 2. J. Gen. Virol. **19**:217–233.
53. **Smith, J. W., E. Adam, J. L. Melnick, and W. E. Rawls.** 1972. Use of the ^{51}Cr release test to demonstrate patterns of antibody response in humans to herpesvirus types 1 and 2. J. Immunol. **109**:554–564.
54. **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. J. Virol. **17**:991–1008.
55. **Spear, P. G., and B. Roizman.** 1980. Herpes simplex viruses, p. 615–745. *In* J. Tooze (ed.), DNA tumor viruses. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
56. **Vestergaard, B. F., and B. Norrild.** 1978. Crossed immunoelectrophoresis of a herpes simplex virus type 1-specific antigen: immunological and biochemical characterization. J. Infect. Dis. **138**:639–643.
57. **Webster, R. G., and B. A. Askonas.** 1980. Cross-protection and cross-reactive cytotoxic T cells induced by influenza virus vaccines in mice. Eur. J. Immunol. **10**:396–401.