Study of Immune Cytotoxicity with *Herpesvirus hominis* Infected Cells[1] (36866)

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A variety of techniques have been used to show that cells appear to acquire new surface antigens upon infection with *Herpesvirus hominis* (HVH) (1–8). Among these techniques is the demonstration of an immune cytotoxic reaction (1, 2). Roane and Roizman (1) reported that infected HEp-2 cells were susceptible to injury by rabbit anti-HVH serum in the presence of complement, since infected cells failed to produce plaques after such treatment. Brier, *et al.* (2) found that Cr^{51} labelled cells released label upon such treatment.

The study reported here used a more direct cytotoxicity test, modified from that of Bases (9) and Hellström and Sjögren (10), to study the cytotoxic phenomenon. This test used failure of infected cells to attach after serum-complement treatment as a measure of immune cytotoxicity. It was found that cells infected with HVH, as well as uninfected cells, settle out of suspensions and attach to the surface of tissue culture dishes within one hour following suspension with trypsin, but infected cells treated with anti-serum and complement are apparently damaged by this treatment and unable to attach.

Materials and Methods. Target cells. The FT-1 line of fetal tonsil fibroblasts (FT) used in passages 12–17 was derived in our laboratory (11). Cells were grown in Eagle's minimum essential medium with 10% fetal-calf serum, 6.6 m*M* sodium bicarbonate, 100 μg/ml streptomycin, 100 IU/ml penicillin and 50 IU/ml mycostatin (MEM). FT monolayers were infected with at least 10 plaque-forming units (PFU) of HVH per cell and the cells harvested with trypsin at 16–20 hr after infection. Variations in multiplicity of infection and time of harvest are described under Results. Target cells were suspended in MEM at a concentration of 6,000 cells/ml. Uninfected FT cells were prepared in a similar manner for use as controls.

Viruses. Four clinical isolates of HVH were used. The origin of type 1 UW-168 and type 2 UW-268 has been described (12). Type 1 E-377 and type 2 E-304 strains were kindly supplied by Dr. A. J. Nahmias of Emory University. Stock viruses for *in vitro* experiments were propagated in FT cells and stored at −80°. UW-168 and UW-268 stocks used for immunization of rabbits were propagated in the SIRC line of rabbit corneal cells.

Serum. Anti-sera (AS) were obtained from rabbits given experimental herpes keratitis by inoculation of the scarified cornea. Rabbits #2 and 4 to 6 were given type 1 UW-168; rabbits #7 to 12 and #17 were given type 2 UW-268. Rabbits #2, 8, 10 and 11 developed signs of encephalitis and were exsanguinated 2–3 weeks after infection. The remaining rabbits received three intramuscular injections of virus in incomplete Freund's adjuvant at 3–4 week intervals after recovery and were bled 10 days after the last booster.

Four rabbit anti-sera were supplied by Dr. Nahmias. Two (X204 and X243) were obtained from rabbits immunized via the corneal route and the other two (Z411B and Z411C) from rabbits immunized with intraperitoneal and intramuscular injections (13). Preimmunization sera from rabbits #5, 11 and 17 or sera from normal uninoculated rabbits were used as controls (CS).

Complement. Normal rabbit sera were used as a source for the heat-labile enhancing factor, which is presumed on the basis of the work of others (1, 14) to be complement. The sera were tested for toxicity to uninfected cells and acceptable sera were pooled and

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stored at −80°.

Cytotoxicity test. Sera were heated at 56° for 30 min, then diluted 1:4 and mixed with equal volumes of a 1:4 dilution of complement. Four 25 μl portions of these mixtures were placed in either a 60 mm tissue-culture dish or in wells of a tissue-culture microtest plate. A 25 μl aliquot of target cells was added to each portion. The final 50 μl reaction mixture contained 1:16 dilution of both test serum and complement plus 100–200 target cells. The dishes or plates were incubated 40–50 min in a 37° humidified incubator in 2.5% CO_2 in air. Infected and uninfected cells were reacted with both control and test sera in each set of experiments. Following incubation (during which time unaffected cells attached to the surface of the containers), the dishes or plates were washed, fixed with 5% formalin and stained with crystal violet. Attached cells were counted with the aid of a stereomicroscope (Stereo-Star/Zoom, American Optical Corp., Buffalo, N.Y.). The four cell counts for each serum were averaged and the standard error computed. The average number of target cells in a serum-complement mixture which attached was compared to the number which attached from a similar inoculum without serum-complement and the percent attachment calculated, using the control average as 100%.

Serum titrations. Serial twofold dilutions of serum were tested for cytotoxicity as described above. Those dilutions producing a 50% or greater reduction in cell attachment were considered as showing cytotoxicity. The cytotoxic titer was determined by the method of Reed and Muench (15) and expressed as the negative logarithm of the 50% cytotoxic end point. Anti-sera that produced less than a 50% reduction in attachment at the initial dilution of 1:16 were designated as having a titer less than 1.2.

Results. Cytotoxicity test. The results of testing the UW rabbit anti-sera against cells infected with the homologous immunizing strains, and the cross-reactivity of each serum with cells infected with the heterologous type is shown in Table I. The number of infected cells which attach after exposure to control and anti-herpes sera is compared to the attachment of uninfected cells under

TABLE I. Cytotoxic Effect of Rabbit Anti-sera on HVH-Infected Cells.

			HVH infected cells			
	Uninfected cells		UW-168 (Type 1)		UW-268 (Type 2)	
Serum	No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]
None	119.8 ± 3.4[b]	100.0	105.3 ± 5.0	100.0	124.3 ± 11.6	100.0
Anti UW-168						
# 2	99.3 ± 3.6	82.9	4.3 ± 0.6	4.1	29.6 ± 3.3	23.8
Anti UW-268						
# 7	97.5 ± 3.0	81.4	5.5 ± 1.3	5.2	22.8 ± 2.2	18.3
8	98.3 ± 3.8	82.1	6.3 ± 1.5	6.0	22.3 ± 1.5	17.9
9	95.8 ± 5.2	80.0	2.0 ± 0.4	2.0	56.3 ± 3.6	45.3
10	106.8 ± 5.9	89.1	3.3 ± 0.8	3.1	25.8 ± 2.4	20.7
11	91.8 ± 4.0	76.6	5.5 ± 1.0	5.2	20.5 ± 2.6	16.5
12	94.3 ± 5.2	78.7	5.0 ± 1.7	4.7	14.5 ± 2.6	11.7
Control						
# 5	102.8 ± 4.5	85.8	80.0 ± 4.3	76.0	93.8 ± 5.0	75.5
11	98.3 ± 5.3	82.1	83.0 ± 7.0	78.8	104.0 ± 6.6	83.7
50	100.8 ± 7.4	84.1	84.3 ± 5.7	80.1	104.5 ± 3.1	84.1
64	102.5 ± 7.3	85.6	83.0 ± 6.1	78.8	88.8 ± 6.2	71.4

[a] Number of uninfected or infected cells which attach in presence or absence of anti-serum and complement; average of quadruplicate cell counts ± standard error.

[b] Cell attachment as percent of control cells in absence of serum and complement.

similar conditions. Control sera reduced the attachment of both infected and uninfected cells by approximately 25%. UW antisera reduced attachment of infected cells by 55–94% without reducing attachment of uninfected cells more than did control sera. None of the sera exhibited type specificity in the cytotoxic test. Three of the anti-sera were tested against cells infected with the two Emory viruses. The UW anti-sera were equally effective with these strains, and reduced the attachment of infected cells significantly (70–97%) without reducing attachment of uninfected cells more than did control sera (25–29%). No type specificity was observed.

The anti-sera produced at Emory by intramuscular and intraperitoneal injections were compared to those produced by corneal inoculation to determine whether the appearance of keratitis has any relation to the development of serum cytotoxicity or its type specificity. Both anti-type 1 sera reduced the attachment of type 1 infected cells to 1–2% and type 2 cells to 13–28% of control plating. The anti-type 2 sera were also equally efficient in reducing attachment of both types of infected cells, although one produced only a 57–61% reduction compared to the 93–97% produced by the more potent anti-serum. The comparisons showed that eye infections are not necessary for the production of cytotoxic

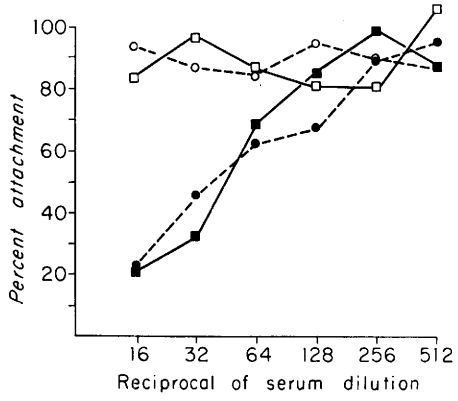

FIG. 1. Effect of dilution on serum cytotoxicity: anti-herpes serum #10 was reacted with UW-168 (●) and UW-268 (■) infected cells. As a control, serum from an uninoculated rabbit, CS#164, was also tested with UW 168 (○) and UW 268 (□) infected cells.

TABLE II. Cytotoxic Titers of Rabbit Anti-HVH Sera.

	Cytotoxic titer to HVH-infected cells	
Rabbit anti-sera	Type 1 UW-168	Type 2 UW-268
Anti-Type 1		
# 2	2.10[a]	1.50
X204	2.40	1.90
Z411C	2.20	1.35
Anti-Type 2		
# 7	1.65	1.50
8	1.65	1.65
9	1.65	1.40
10	1.65	1.65
11	1.95	2.20
12	2.25	2.40
X243	2.50	2.50
Z411B	1.20	1.20

[a] Titer as $\log_{10}$ of the 50% end point.

anti-sera and that anti-sera from animals inoculated by other routes are no more type specific than those from animals with keratitis.

Anti-serum titrations. When serial dilutions of anti-sera were tested, attachment of cells increased progressively wtih serum dilution until it reached the levels produced with control sera, as shown in Fig. 1, where percent attachment is plotted against dilutions of AS#10 and CS#164. Similar dilution of the control serum did not decrease its effect upon attachment, since the amount of unheated normal rabbit serum (complement) remained constant and produced a nonspecific reduction.

The $\log_{10}$ of the 50% end point titers of the anti-sera are shown in Table II. Although some of the type 2 anti-sera had higher titers against type 2 than against type 1 infected cells, some had equal titers for both types and some had higher titers against the type 1 infected cells. The three type 1 anti-sera had higher titers for type 1 than for type 2 infected cells. The $\log_{10}$ cytotoxic titers ranged from 1.2 to 2.5.

Complement-dependency of the cytotoxic reaction. The cytotoxic reaction was found to be totally dependent upon a heat-labile factor in normal sera, presumed to be comple-

TABLE III. Complement-Like Factor Dependency in the Cytotoxic Reaction.

Rabbit serum	Average count ± SE[a]	% Attachment
None	118.0 ± 5.1	100.0
Heated complement	95.8 ± 3.8	81.2
Fresh complement	92.8 ± 4.5	78.6
Heated #11	93.0 ± 5.1	78.8
Heated #11 plus heated complement	91.5 ± 3.5	77.5
Heated #11 plus fresh complement	7.8 ± 1.5	6.6

[a] Average of quadruplicate cell counts ± standard error.

ment, as described in previous reports of this phenomenon (1, 2). As shown in Table III, omission or heating of complement eliminated the cytotoxic effect. Anti-serum alone, or anti-serum plus heated complement reduced attachment by about 20%, while anti-serum plus unheated complement produced a 93% reduction.

When serial twofold dilutions of complement were mixed with serial twofold dilutions of AS#17 in reactions with UW-268 infected cells, the cytotoxic titer of the anti-serum

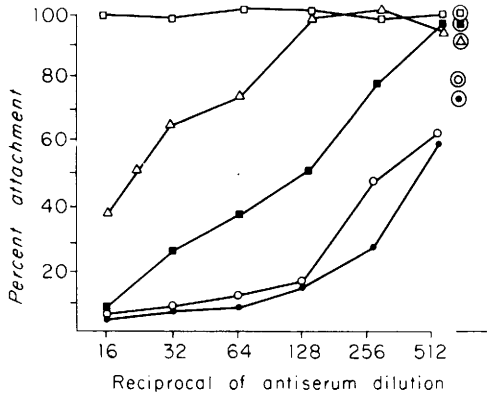

FIG. 2. Effect of complement concentration on anti-serum titer: serial 2-fold dilutions of complement were mixed with serial 2-fold dilutions of anti-herpes serum #17 and reacted with UW-268 infected cells. The percent of cell attachment with antiserum dilutions of 1:16 to 1:512 in the presence of complement diluted 1:10 (●), 1:20 (○), 1:40 (■), 1:80 (△), and 1:320 (□) are shown. Encircled symbols represent the action of complement dilutions tested without anti-serum.

was markedly affected by the complement concentration (Fig. 2). Low dilutions of complement were somewhat cytotoxic without anti-serum and this effect was lost only when a 1:40 dilution was reached. However, complement at this concentration was not sufficient for full expression of the cytotoxic titer of the anti-serum. Since 1:10 and 1:20 dilutions of complement gave very similar titration curves with anti-serum and approximately the same 50% cytotoxic end points, a 1:16 dilution of complement was used in the cytotoxicity test.

Relation of cytotoxicity to multiplicity of infection (MOI). To determine whether the extent of cytotoxicity was directly proportional to the amount of infecting virus, FT cells were inoculated with varying multiplicities of infection, ranging from 3×10^{1} to 3×10^{-4} PFU of UW-268 per cell. Target cells were harvested at 20 hr after infection and reacted with AS#17 and its preimmune serum, CS#17. Percent attachment is plotted against MOI in Fig. 3. These results suggest that susceptibility to cytotoxicity is directly related to input MOI and presumably therefore to the number of infected cells. It appears, however, that the calculated input MOI either does not accurately indicate the true number of infected cells or that not all infected cells are susceptible to the cytotoxic effect, since the zero class of the Poisson distribution for the higher multiplicities would indicate that fewer uninfected cells should be present than were found to attach. At an input MOI of 0.003, no significant number of infected cells would be expected and at this point anti-serum had no more effect upon the cells than did control serum.

Post-infection time study. The time at which cells develop susceptibility to cytotoxic anti-sera was studied by harvesting UW-268 infected cells at time periods ranging from 1 to 12 hr after infection and reacting them with AS#11 and its preimmune serum, CS#11. The results of two experiments are shown in Fig. 4, where percent attachment is plotted against post-infection time in hours. No significant reduction in attachment was seen in the first 4 hr after infection. A 50% reduction occurred at 5–6 hr and attachment continued to decline thereafter. It was con-

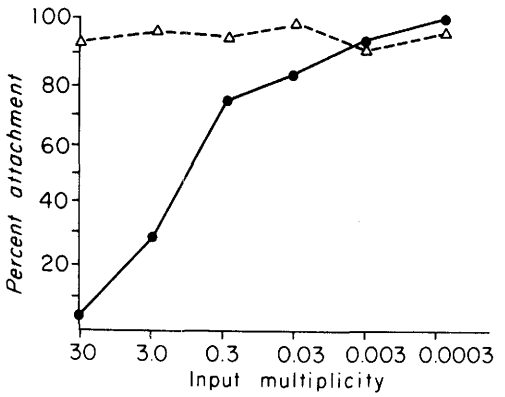

FIG. 3. Effect of multiplicity of infection on susceptibility to cytotoxicity: FT cells were inoculated with varying multipicities of infection of UW-268 and the harvested cells reacted with anti-herpes serum #17 (●) and preimmune control serum (△).

cluded that changes in infected cells which render them susceptible to immune cytotoxicity are not manifested during the first 4 hr after infection.

Discussion. The role of oncogenic viruses in conferring new antigenic specificity upon the surface of neoplastic cells is well known (16, 17) and a number of viruses not known to be oncogenic, in addition to herpesviruses, also appear to induce virus-specific antigens on the cell surface. This phenomenon has been proposed for agents from at least six different taxonomic groups with highly divergent properties. These include both DNA-containing viruses such as herpes (1–8) and pox (18, 19) as well as RNA-containing viruses, such as myxo- (20, 21), arbo- (22), rhabdo- (23) and arenoviruses (24).

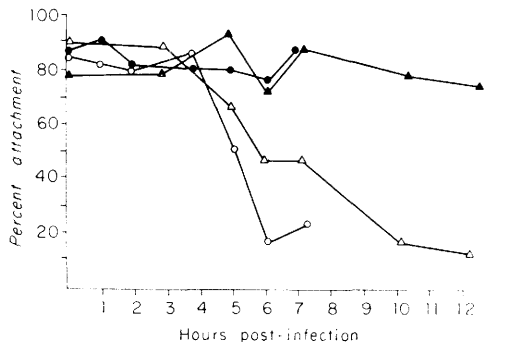

FIG. 4. Temporal development of cytotoxic susceptibility in infected cells: UW-268 infected cells were harvested at intervals 1 to 12 hr after infection and reacted with anti-herpes serum (○△) and control preimmune serum (●▲). Results of two experiments are shown: Exp. #1 = ○ ●; Exp. #2 = △▲.

The acquisition of new surface antigenicity by HVH-infected cells has been studied by means of cytotoxicity tests (1, 2), cell membrane isolation (3, 4), ferritin-conjugation (5), hemadsorption (6), immunofluorescence (7) and mixed agglutination (8) techniques. This report presents evidence that HVH-infected cells have an altered immune specificity and are subject to damage by anti-HVH sera in the presence of complement. Infected cells which did not attach but remained suspended in the anti-serum-complement mixture were found to be swollen, to lack refractibility and to take up trypan blue dye. By these criteria, they may be presumed dead. The cytotoxicity reaction exhibited no type specificity and it would appear that the surface of type 1 and type 2 infected cells share a common antigen or antigens.

Although no firm conclusions can be drawn from the small number of sera tested, these type 2 anti-sera showed greater cross-reactivity than did the type 1 anti-sera. The tendency of type 2 anti-sera to have comparable cytotoxic titers for cells infected with either type is similar to the results obtained by microneutralization techniques (13).

Absence of cytotoxic susceptibility during the first four hours after viral infection argues against the possibility that new surface antigens may be due to either attached virions or discarded envelopes from penetrating virions. The appearance of susceptibility at 5–6 hours after infection suggests that egressing virus cannot be responsible for the cytotoxic effect. The data from one-step growth studies with HVH in human embryonic lung fibroblasts (25), as well as from unpublished studies with FT cells, suggest that virus is not released so early in the infection cycle. The onset of cytotoxic susceptibility appears to coincide with the earliest appearance of densely cored and enveloped particles within the infected cell (26, 27).

The role of immune cytotoxicity in host defense against herpesvirus infection remains

to be determined. It is conceivable that this mechanism operates *in vivo* and serves to prevent viral replication and spread. Herpesviruses readily produce latent infections with recurrent exacerbations and immune cytotoxicity may be part of the host defense which prevents the development of generalized infection from recurrent local lesions, since herpes lesions are known to occur in the presence of circulating antibody.

Summary. Immune cytotoxicity of herpesvirus-infected cells was studied by means of a direct cytotoxicity test which used failure to attach after treatment with antiviral serum and complement as a measure of cytotoxicity. Cells infected with herpesvirus, as well as uninfected cells, were found to settle out of suspension and attach to the surface of containers within one hour after suspension. Infected cells appeared to be damaged by serum-complement treatment and were unable to attach. Immune cytotoxicity was found to be complement-dependent, since either omission or heating of the normal rabbit serum used as a source of complement eliminated the cytotoxic effect. Cell susceptibility to cytotoxic effects was found to be directly related to viral multiplicity of infection and presumably, therefore, to the proportion of infected cells. Infected cells were not susceptible during the first four hours after viral infection, but developed this susceptibility within 5–6 hours after infection. Anti-serum produced by either corneal or intramuscular routes of inoculation exhibited cytotoxic activity. Cytotoxic titers of antiserum were calculated as the $\log_{10}$ of the highest dilution of serum producing a 50% reduction in cell attachment, and titers of eleven rabbit anti-sera varied from 1.2 to 2.5. The cytotoxic reaction was not type specific and anti-sera produced against either type 1 or type 2 herpesvirus exhibited cytotoxicity for cells infected with either type.

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