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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

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Tissue culture cells infected with herpes simplex virus type 1 synthesize three major glycoprotein antigens (Ag-11, Ag-8, and Ag-6), which have been characterized by crossed immunoelectrophoresis. The three viral antigens have been identified as a mixture of gA and gB (Ag-11), gD (Ag-8), and gC (Ag-6). Recent findings have shown that antibodies directed to each of the three antigens individually are able to mediate antibody-dependent cell-mediated cytotoxicity when tissue culture cells late in the infectious cycle (18 h postinfection) are used. In this work, antibody-dependent cell-mediated cytotoxicity was applied to study the time postinfection at which the individual viral antigens first made their appearance at the cell surface. All three viral antigens (Ag-11, Ag-8, and Ag-6) could be demonstrated as newly synthesized from 3 to 4 h postinfection, and the quantities of the antigens at the surfaces of the infected cells increased with time postinfection. The use of cycloheximide and ultraviolet-inactivated virus demonstrated that input virus could be detected by antibody-dependent cell-mediated cytotoxicity during the first 2 h postinfection, but the cytotoxicity caused by input virus remained constant with time postinfection. In conclusion, these observations demonstrate the participation of individual herpes simplex virus surface antigens in antibody-dependent cell-mediated cytotoxicity attack on cells early in infection.

Infection of tissue culture cells with herpes simplex virus type 1 (HSV-1) is known to induce changes in the plasma membrane of the host cells. Virus-specific proteins have been demonstrated by a variety of methods (7, 12, 13, 15), and it has been shown that of the viral proteins, it is mainly the glycoproteins that are exposed on the surfaces of infected cells (4, 8). By crossed immunoelectrophoresis of extracts of HSV-1-infected cells, three viral glycoprotein antigens have been demonstrated consistently, which are designated Ag-11, Ag-8, and Ag-6; of these, Ag-6 has been shown to be HSV-1 specific (21). The antigens have been correlated to the glycoproteins identified by other investigators, who designated the four glycoproteins gA, gB, gC, and gD (18). Ag-11 corresponds to a mixture of gA and gB, Ag-8 corresponds to gD, and Ag-6 corresponds to gC (9, 10).

It has been demonstrated previously that tissue culture cells infected with HSVs can be lysed either by convalescent human sera positive for HSV-1 antibody or by rabbit hyperimmune sera obtained by infection of rabbits with HSV-1 in the presence of various types of peripheral blood leukocytes (K-lymphocytes, polymorphonuclear leukocytes, or monocytes) which have been obtained from individuals independent of their previous exposure to HSV (6, 11, 15). This immune mechanism, termed antibody-dependent cell-mediated cytotoxicity (ADCC), is a very sensitive mechanism for detecting HSV antibodies. By using antibody preparations directed mainly to one of the glycoprotein antigens Ag-11, Ag-8, and Ag-6, recent studies have demonstrated that each of these antigens is exposed at the surface of the plasma membrane of HSV-1-infected cells late in the infectious cycle and is capable of participating in two different cytolytic processes, ADCC and antibody-dependent complement-mediated lysis (10). In the present study the major conclusions are that (i) the individual viral glycoprotein antigens are demonstrable early in the infectious cycle by employing antibodies directed mainly to Ag-11, Ag-8, and Ag-6 in the ADCC test, and (ii) the immediate early ADCC reaction is caused by input virus and can be differentiated from the reaction caused by

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newly synthesized viral glycoproteins by employing ultraviolet (UV)-inactivated virus or the protein inhibitor cycloheximide (CH).

MATERIALS AND METHODS

Tissue culture cells. Chang liver cells were grown and maintained as previously reported (10). These cells were used instead of HEp-2 cells, since recent findings have demonstrated better surface expression of the Ag-6 glycoprotein antigen of HSV-1 (10).

Virus infection. (i) Analysis of antigen expression at 18 h postinfection. Chang liver cell monolayers grown in 25-cm^2 plastic flasks were inoculated with HSV-1 strain VR-3 (MacIntyre) at a multiplicity of infection of 1 to 2 plaque-forming units per cell in a final volume of 0.3 ml of Eagle minimum essential medium (MEM) containing 2% fetal calf serum (FCS). After adsorption of virus at 37°C for 45 min, 10 ml of MEM containing 2% FCS was added to the culture. After 18 h cells were suspended with 0.25% trypsin containing 0.05% ethylenediaminetetraacetate and washed twice, and 5×10^6 cells were labeled for 1 h at 37°C with 100 μCi of ^{51}Cr in a final volume of 1.0 ml. The cells were centrifuged through a 4-ml cushion of FCS inactivated at 56°C for 30 min (I-FCS). The cell pellet was then suspended in MEM containing 10% I-FCS.

(ii) Analysis of surface antigen expression at 0 to 10 h postinfection. Monolayers of Chang liver cells grown in 25-cm^2 flasks were labeled for 1 h with 100 μCi of ^{51}Cr on the day before the experiments were performed and overlaid with 10 ml of MEM containing 2% FCS. The labeled cells were suspended with trypsin as described above and washed twice. The cell pellet (2×10^6 to 5×10^6 cells) was inoculated with HSV-1 at a multiplicity of infection of 15 plaque-forming units per cell in a final volume of 0.5 to 1.0 ml. After 45 min of viral adsorption at 37°C, cells were washed three times at 4°C in MEM containing 2% I-FCS. Cells were resuspended (zero time) in MEM containing 10% I-FCS when used immediately in ADCC and in MEM containing 2% I-FCS when used later in the infectious cycle. In the latter case, the cells were incubated at a cell density of 10^6 cells per ml at 37°C in a CO_2 incubator until used. Cells were then sampled at different times postinfection, washed once at 4°C, and resuspended in MEM containing 10% I-FCS for the ADCC assay.

UV-inactivated virus was prepared by exposure of the virus stock for 5 min to a 30-W germicidal lamp at a distance of 10 cm. No infectious virus was recoverable after this treatment.

For inoculation purposes, a volume of UV-inactivated virus equivalent to the volume used for standard virus was added to the cell pellet.

Effector cells. Human mononuclear effector cells (MC) were prepared from the heparinized blood of healthy adult donors by centrifugation on Ficoll-Hypaque gradients (3, 15). Cells at interphase were washed three times in Hanks balanced salt solution as described previously (15), and they were resuspended in MEM containing 10% I-FCS.

Antibodies. Polyspecific antibodies of HSV-1 were raised in rabbits as previously reported (20). Antibodies reacting to Ag-11, Ag-8, and Ag-6 were raised by immunization of rabbits with immunoprecipitates cut from agarose gels after crossed immunoelectrophoresis of a Triton X-100-solubilized HSV-1 antigen prepared from infected rabbit kidney cells. The globulin fraction was purified from all rabbit sera (including preimmune sera) by the method of Harboe and Ingild (2). The protein concentration was 35 to 40 mg/ml. Samples (1 ml) of each of the rabbit globulin preparations were absorbed three times with cell pellets (5×10^7 cells) of uninfected Chang liver cells. Antibody preparations were heat-inactivated at 56°C for 30 min.

ADCC. All ADCC assays were performed in a reaction volume of 1.0 ml of MEM containing 10% I-FCS in plastic culture tubes (12 by 75 mm) at 37°C in 95% air–5% CO_2; 2×10^4 HSV-1-infected Chang liver cells or uninfected cells as controls were mixed with an appropriate number of MC, as described in the legends to the figures. Two ADCC techniques were employed. In the first, the one-step method, the different globulin preparations were added directly to the mixture of target cells and MC. In the second, the two-step method, target cells (5×10^5) were presensitized at room temperature for 15 min with 0.5-ml amounts of the different globulin preparations and washed three times at 4°C to remove unbound antibodies. After resuspension in MEM containing 10% I-FCS, the presensitized targets were mixed with MC. The dilutions of the globulin preparations employed and the times of incubation for both of the ADCC methods are specified in the figure legends. Release of ^{51}Cr by the target cells into the medium was determined after centrifugation at $300 \times g$ for 15 min as previously described (15). The ^{51}Cr release was estimated as $2A/(A + B) \times 100$, where A was the counts per minute in the top 0.5 ml and B was the counts per minute in the bottom 0.5 ml. The samples were counted in a Beckman gamma counter. The percent specific release was defined as the percent ^{51}Cr release from target cells exposed to MC and immune globulin minus the percent ^{51}Cr release from target cells exposed to MC in the absence of specific antibodies by the use of nonimmune globulin (Table 1). All assays were performed in triplicate. The maximal cell-associated isotope release after three freeze-thaw cycles averaged 85%. Table 1 shows the amounts of isotope released from infected and uninfected target cells exposed to MC and to MC plus nonimmune globulin. Antibody titers were estimated as reciprocal of the dilution yielding one-half of the maximal specific release obtained from cells infected for 18 h (19).

Chemicals and radioisotopes. CH was obtained from Sigma Chemical Co., St. Louis, Mo., and ^{51}Cr (sodium chromate; 200 to 500 Ci/g of chromium) was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Titration of the antibody preparations. To standardize the amounts of antibodies used for each preparation, the antibodies were titrated by using the one-step ADCC method. HSV-1-infected cells were harvested 18 h postinfection, labeled, and exposed to MC at a ratio

of effector to target cells of 30:1 in the presence of immune globulins in dilutions ranging from 1:1,500 to 1:500,000 (Fig. 1).

The polyspecific antibodies and the three different antibody preparations (anti-Ag-11, anti-Ag-8, and anti-Ag-6) gave similar plateau values of percent specific release when used at dilutions of up to 1:5,000. For polyspecific antibodies and anti-Ag-8 the plateau extended to dilutions of 1:15,000 and 1:50,000, respectively, whereas the antibody preparations to Ag-11 and Ag-6 gave decreasing percent specific release when diluted beyond 1:5,000. The titers measured as the 50% endpoints were as follows: polyspecific anti-HSV-1, 47,000; anti-Ag-11, 34,000; anti-Ag-8, 325,000; and anti-Ag-6, 40,000. For the following studies related to the time of expression of individual surface antigens, as assessed by the one-step ADCC method, the different globulin preparations were employed at concentrations 100 times greater than their 50% endpoint titers.

TABLE 1. *Specificity of the ADCC reaction*[a]

Antibody	% ^{51}Cr release		
	HSV-1-infected cells reacted at:		Mock-infected cells
	Zero time[b]	18 h postinfection[c]	
None	10.2	5.5	12.0
Nonimmune rabbit globulin	9.4	9.4	11.1
HSV-1 polyspecific rabbit globulin	29.8	56.2	11.7
^{51}Cr specific release	20.4	46.8	0.6

[a] Each value is the mean of two experiments in which each determination was a mean of three parallel estimations. Incubation time was 4 h.
[b] Multiplicity of infection, 15:1.
[c] Multiplicity of infection, 1:1.

Specificity of the ADCC reaction. The percent ^{51}Cr release was measured by employing rabbit nonimmune and immune globulins to either HSV-1-infected or to mock-infected cells. Table 1 shows a significant increase in ^{51}Cr release when polyspecific HSV-1 antibodies were employed to HSV-1-infected cells. Polyspecific HSV-1 antibodies did not react to mock-infected cells, a finding which demonstrates the specificity of the ADCC test. The low percent ^{51}Cr release measured in infected cells in the presence of nonimmune globulins and in mock-infected cells shows that suspension of the monolayer cells with trypsin-ethylenediaminetetraacetate did not destroy the cells. The influence of the trypsin treatment on the HSV-1 antigens was studied in two different ways. Infected cells were employed in the ADCC test either at 18 h postinfection (infected cells were suspended with trypsin-ethylenediaminetetraacetate) or at zero time (cells were suspended before infection, washed twice, and then infected at a multiplicity of infection of 15:1). After adsorption and extensive washing of the infected cells, they were incubated with antibodies and effector cells. A significant percent specific ^{51}Cr release was obtained with both sets of target cells (Table 1). It should be stressed that the viral antigens on zero time-infected cells were not exposed to trypsin at all.

Kinetic analysis of HSV-1 surface antigens in the first 8 h postinfection. (i) One-step ADCC. To measure the time sequence of the surface expression of Ag-11, Ag-8, and Ag-6, we first used the one-step ADCC system. HSV-1-infected Chang liver cells were mixed with MC and antibodies at 0, 1, 2, 4, and 6 h postinfection. Initially, the samples were centrifuged for 2 min at 1,500 rpm in order to accelerate the ADCC

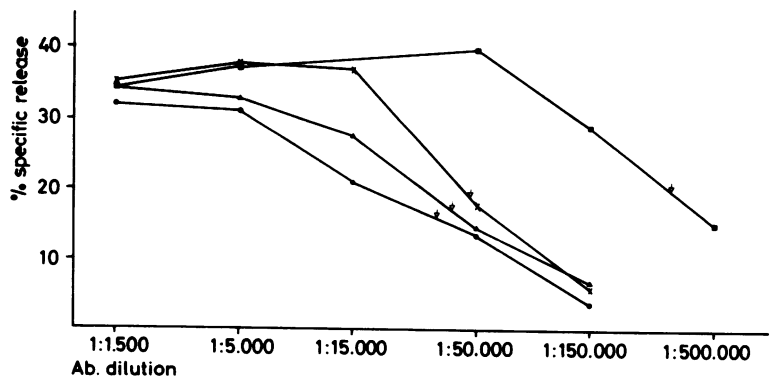

FIG. 1. *Titration of immune globulin preparations in ADCC against HSV-1-infected target cells (18 h postinfection). The ratio of effector cells to target cells was 30:1. Symbols: ×, polyspecific anti-HSV-1; ●, anti-Ag-11; ■, anti-Ag-8; ▲, anti-Ag-6. Arrows represent the antibody (Ab.) titers of the various immunoglobulins.*

reaction by eliminating the lag period in the initiation of the cytolytic reaction (14). After incubation at 37°C for an additional 2 h, the samples were harvested, and the specific ^{51}Cr release was measured (Fig. 2). The results represent the percent specific release as a function of time of harvest postinfection.

The polyspecific sera to HSV-1, as well as each of the antibody preparations to Ag-11, Ag-8, and Ag-6, were found to initiate ADCC within 2 h postinfection. With all antibodies, cytotoxicity increased steadily thereafter. The anti-Ag-11 globulin yielded a reaction similar in magnitude to that of the polyspecific globulin. Although the anti-Ag-8 and anti-Ag-6 preparations produced somewhat lower levels of cytotoxicity, the slopes of the kinetic reaction curves were similar for all of the immune globulins.

(ii) Two-step ADCC. Figure 3 shows the results obtained when the two-step ADCC system was employed with target cells at 2, 3, 4, 6, and 8 h postinfection. This technique allows a more precise determination of the time at which individual viral antigens appear at the cell surface, since the reaction measures the recognition of surface antigens during only a 15-min exposure to antibodies. A uniform dilution of all immune globulins (1:100) in considerable antibody excess was employed because the two-step method with polyspecific antibodies had previously been found to be less sensitive than the one-step assay (17). Since excess antibodies are

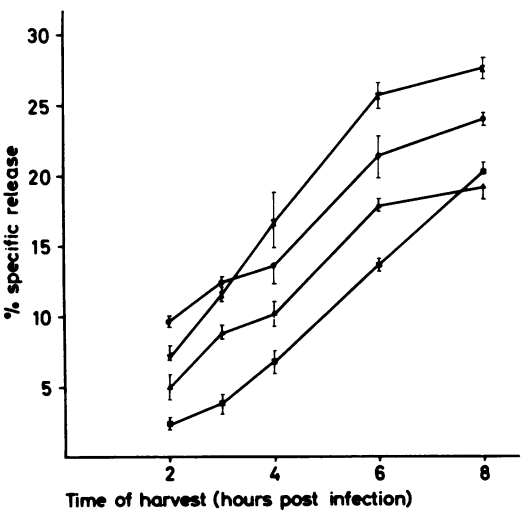

FIG. 2. *Time of detection of HSV surface antigens as analyzed by the one-step ADCC assay. The ratio of effector cells to target cells was 50:1. All plotted data are means ± standard errors of the mean of triplicate determinations. Symbols: ×, polyspecific anti-HSV-1; ●, anti-Ag-11; ■, anti-Ag-8; ▲, anti-Ag-6.*

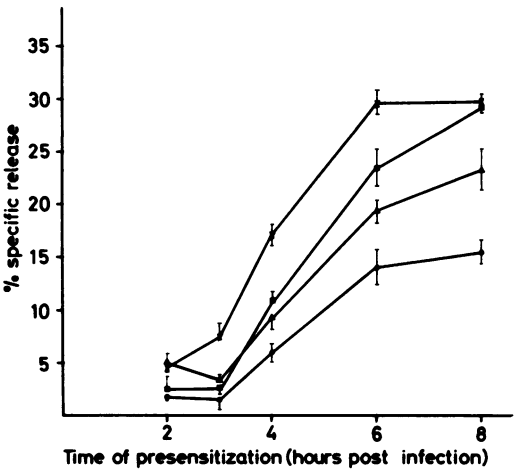

FIG. 3. *Time of detection of HSV surface antigens as analyzed by a two-step ADCC assay, using a 4-h incubation period. All rabbit globulins were used at a 1:100 dilution. The ratio of effector cells to target cells was 50:1. All plotted data are means ± standard errors of the mean of triplicate determinations. Symbols: ×, polyspecific anti-HSV; ●, anti-Ag-11; ■, anti-Ag-8; ▲, anti-Ag-6.*

removed, the additional 4 h of incubation used to detect ^{51}Cr release does not influence the time of actual surface antigen detection. The percent specific release increased significantly from 4 to 6 h postinfection; this was followed by a smaller increase from 6 to 8 h postinfection. It should be noted that a small amount of cytotoxicity (≤5%) was measurable at 2 to 3 h postinfection with all immune globulins employed.

Effect of CH on surface antigen expression. The mediation of ADCC by viral antibodies very early postinfection might represent reaction with input virus rather than with newly synthesized viral surface antigens. To examine this possibility, we studied the effect of inhibition of viral protein synthesis by CH on the kinetics of the ADCC reaction during the first 10 h of infection. It has been found previously that CH has no influence on the ability of human effector cells to mediate ADCC (16). The effects of CH on the kinetics of ADCC mediated by the three antibody preparations to Ag-11, Ag-8, and Ag-6 are shown in Fig. 4. In this one-step assay, target cells in the presence or absence of CH were exposed to MC and antibodies immediately after viral adsorption. The kinetics of specific ^{51}Cr release were measured at various intervals during the ensuing 10-h incubation period.

The plateau values of the percent specific release mediated by all three antibody preparations were significantly reduced in the presence of CH (Fig. 4A). The reaction mediated by anti-

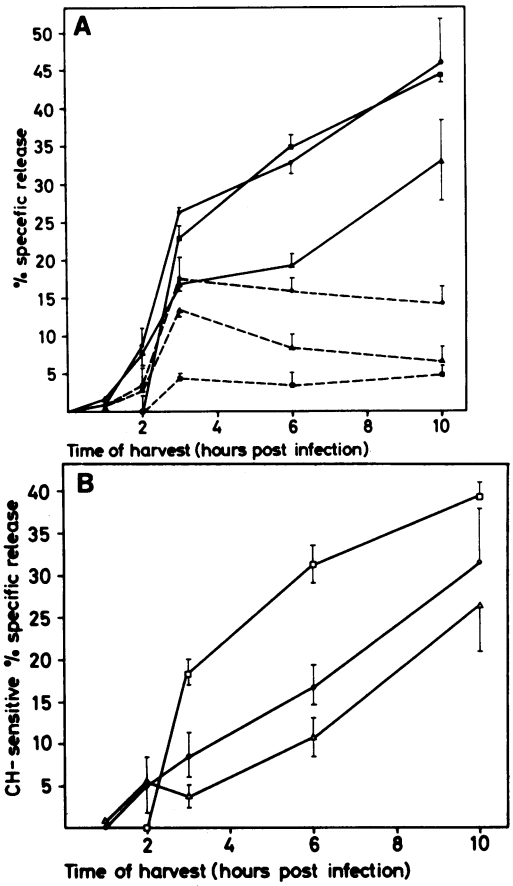

FIG. 4. *Effect of CH on the expression of the HSV surface antigens in a one-step kinetic ADCC assay. The ratio of effector cells to target cells was 50:1. All plotted data are means ± standard errors of the mean of triplicate determinations. (A) Data are expressed as percent specific release in the presence or absence of CH (50 μg/ml). Symbols: ●——●, anti-Ag-11; ●- - -●, anti-Ag-11 + CH; ■——■, anti-Ag-8; ■- - -■, anti-Ag-8 + CH; ▲——▲, anti-Ag-6; ▲- - -▲, anti-Ag-6 + CH. (B) Data are expressed as percent specific release (expressed as the difference between the release measured in the absence of CH and that measured in the presence of CH). Symbols: ○, anti-Ag-11; □, anti-Ag-8; △, anti-Ag-6.*

Ag-8 or anti-Ag-6 was more completely inhibited than that mediated by anti-Ag-11. The reaction curves of all three antibody preparations in the presence of CH reached plateau values at 3 h postinfection, indicating that ^{51}Cr release was complete after only 3 h of incubation. In contrast, the cytotoxicity observed in the absence of the protein inhibitor increased steadily until 10 h postinfection. Because of the CH-resistant background of cytotoxic activity, the cytotoxicity caused by de novo synthesized viral antigens is obtained by subtracting the CH-resistant background from the cytotoxicity measured in the absence of the CH. As Fig. 4B shows, de novo synthesized Ag-11, Ag-8, and Ag-6 are demonstrable within 3 h postinfection.

Kinetic analysis of target cells exposed to UV-inactivated virus. The contribution of input virus to the ADCC reaction was further explored in experiments in which UV-inactivated virus was employed in the test. The percent specific ^{51}Cr release was measured in a one-step ADCC kinetic experiment in which the target cells were exposed to UV-inactivated virus and reacted with polyspecific serum and MC at the end of the adsorption period (Fig. 5). A low level of cytotoxicity, which was nonetheless significant, was demonstrated and reached a plateau value at 2 h postinfection. For comparison, the percent specific release from target cells infected with native virus incubated in the presence or absence of CH was measured. The target cells infected with native virus in the absence of CH showed a biphasic reaction curve which reached a relative plateau between 1 and 2 h postinfection, after which an increase in specific release was detected; this increase was dependent on the presence of antibodies reacting to viral antigens. In the presence of CH, the plateau value was reached at 2 h postinfection. The plateau value was similar to that obtained with target cells exposed to UV-inactivated virus. In order to study the specific release as early as 30 min postinfection, all samples were centrifuged to avoid any lag in the initiation of the cytolytic effect.

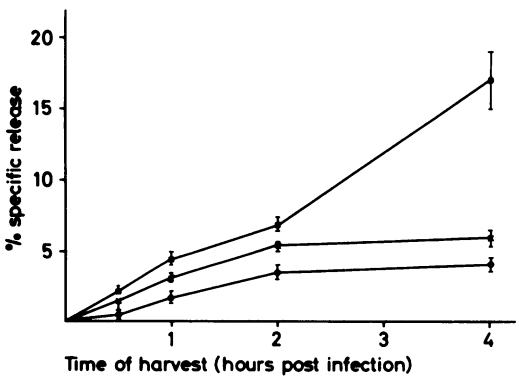

FIG. 5. *One-step kinetic ADCC assay for target cells exposed to UV-inactivated virus compared with target cells infected with native virus in the presence or absence of CH. The ratio of effector cells to target cells was 50:1. All plotted data are means ± standard errors of the mean of triplicate determinations. Symbols: ●, target cells exposed to UV-inactivated HSV; ■, target cells infected with native HSV; ×, target cells infected with native HSV in the presence of CH (50 μg/ml).*

DISCUSSION

Virus-specific antigens have been demonstrated on the surfaces of HSV-infected cells by a variety of methods (7, 13, 15). Of the tests in use, the ADCC reaction has been shown to be the most sensitive in detecting small amounts of either HSV-1 antigens or antibodies to HSV-1 (10, 16, 17). In this work we focused on the specificity of the ADCC reaction and on the demonstration of HSV-1 antigens present at the surfaces of infected cells early postinfection, information which is of importance in determining whether immune cytolytic reactions might be able to lyse infected cells before new progeny can be spread from cell to cell.

The main conclusions obtained are the following. (i) The de novo synthesized individual antigens Ag-11 (gA plus gB), Ag-8 (gD), and Ag-6 (gC) are demonstrable in the plasma membranes of infected cells 3 to 4 h postinfection. And (ii), the small reproducible specific cytotoxicity detectable from 0 to 2 h postinfection is due to antigens from input virus.

The ADCC reaction was shown to be specific, as HSV-1-infected target cells (late as well as early postinfection) were lysed in the presence of immune globulins to HSV-1 and mock-infected cells were not lysed when immune globulins were used in the test.

The kinetic studies performed showed that the antigens Ag-11, Ag-8, and Ag-6 were demonstrated simultaneously 3 h postinfection in the plasma membranes of the infected cells as the percent specific ^{51}Cr release increased from 3 to 10 h postinfection (Fig. 2 and 3). Addition of CH, an inhibitor of protein synthesis, to the ADCC reaction verified that newly synthesized antigens appeared 3 to 4 h postinfection, as the increase in percent specific ^{51}Cr release was inhibited by the presence of CH (Fig. 4A and B). These findings are consistent with the previous demonstration of the synthesis of structural viral proteins early postinfection (5). A coordinated regulation of the viral proteins was also demonstrated in previous studies, and the synthesis of structural viral γ-proteins was dependent on the synthesis of two other groups of viral proteins, α and β (5).

The addition of CH to infected cells inhibited the synthesis of viral proteins completely. Therefore, we concluded that the percent specific ^{51}Cr release resistant to CH represented the presence of viral antigens originating from input virus particles. The reason for the difference in the level of CH-resistant ^{51}Cr release in the presence of anti-Ag-11, anti-Ag-8, or anti-Ag-6 is not known at present (Fig. 4A). It should be mentioned that human antibodies to HSV-1 did not show a similar level of CH-resistant ^{51}Cr release (16). The most likely explanation for this is that the rabbit immune globulins have a higher avidity to the HSV-1 antigens than human antibodies; this is why the rabbit immune globulins are capable of detecting even the very small amounts of viral antigens originating from input virus. The immune cytolytic reaction is an all or none destruction of the infected cells, and binding of specific antibodies to the antigens from the input virus thus initiates cytolysis.

That the percent specific ^{51}Cr release measured before 3 h postinfection is due to antigens originating from the input virus particles is further supported by the following observations.

(i) The kinetic specific release curves (Fig. 3) plateau between 2 and 3 h postinfection for each of the immune globulins, after which an increase in cytotoxicity occurs in parallel to the appearance of de novo synthesized antigens.

(ii) In the presence of CH, a plateau is reached at 3 h postinfection for the kinetic specific release curves of each of the immune globulins (Fig. 4A).

(iii) The kinetic curves for target cells exposed to UV-inactivated virus and for target cells infected with native virus in the presence of CH show plateauing of cytotoxicity by 2 h postinfection.

There are several questions raised by these observations. One problem is the possible contribution of input virus in studies aimed at demonstrating the earliest appearance of surface changes related to HSV (1). Another is whether the adherence of input virus to the cell is purely mechanical or whether it might be related to the plasma membrane, thus providing the possible target for very early specific immunological attack. The possible contiguity of viral envelope antigens and host cell antigens has not been explored yet.

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