Determination of Virus-Specific Antigens in Extracts from Herpes Simplex Virus-Infected Cells by a $^{51}$Cr Release Inhibition Test

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A $^{51}$Cr release inhibition test (CRIT) was developed for determining herpes simplex virus type 1 antigens in infected cell extracts. Among the factors studied were the optimal concentrations of complement, antibody, and infected target cells. Under standardized conditions, the relationship between antigen content and inhibition of chromium release was linear in a semilogarithmic plot, indicating that the antigen content can be determined from testing two dilutions of a given preparation. Other tests showed that infected cells cryopreserved at $-70^\circ$C for periods exceeding 3 months can be used in CRIT. Five different batches of infected cell extracts were tested in both CRIT and blocking neutralization tests with comparable results being obtained, suggesting that both tests measure the same activity.

Recently, efforts to develop herpes simplex virus (HSV) vaccines received new stimuli because of the suspected role of this virus in human cancer. From the point of view of safety, the most reasonable approach to the development of an HSV vaccine probably is to produce a subviral preparation, as discussed in a previous paper (3). Rapid determination of the herpesvirus antigen content in such a preparation is one of the problems which the research worker faces. In our previous experiments the content of antigens reactive with antibodies neutralizing HSV was measured by a blocking neutralization test (3).

Vasconcelos-Costa (13) and Luborsky et al. (4) described a cytotoxicity inhibition test for detection of solubilized surface antigens of cells transformed by adenovirus 12 or Simian virus 40. The present study was undertaken to develop a cytotoxicity inhibition test for determining the content of solubilized HSV antigens. In preparing the test, advantage was taken of the finding that HSV structural antigens appear on the surface membranes of HSV-infected cells (9).

MATERIALS AND METHODS

Cell cultures. Human diploid cells (LEP) and rabbit embryo fibroblasts (REF) were the same as in previous experiments (7, 10). Both cell lines were cultivated in modified medium 199 (EPL) containing calf serum growth-active proteins (6). LEP cells were maintained in medium MEM supplemented with 5% inactivated calf serum; REF cells were maintained in EPL medium.

Virus. Laboratory HSV type 1 (HSV-1) (strain KOS) was kindly provided by J. L. Melnick (Baylor College of Medicine, Houston, Tex.). Virus was grown in human diploid LEP cells at a multiplicity of infection below 1 plaque-forming unit per cell.

Neutralization test. Gamma globulin or HSV-1-hyperimmune serum was diluted in twofold steps starting from 1:10. Equal amounts (0.4 ml) of serum dilution, virus suspension (400 50% tissue culture infective doses), and medium without or with complement (100 U; complement unit determined by the microtechnique described by Závadová et al. [15]) were mixed and incubated at $37^\circ$C for 1 h. Each mixture (0.3 ml) was inoculated into four tube cultures of LEP cells. The test was read after a 6-day incubation at $37^\circ$C. Serum antibody titers were determined according to Kärber (2). The blocking neutralization test was performed as described elsewhere (3).

Gamma globulin and immune serum. Normal human gamma globulin (16% solution) was used. Its stock solution was kept at $-30^\circ$C. The neutralizing titer of complement-independent antibodies against HSV-1 was 1:900, and that of complement-dependent antibodies was 1:2,168. Immune serum was prepared in rabbits by a series of seven injections administered intradermally, intraperitoneally, and intravenously, in 3- to 7-day intervals. HSV-1, strain Kupka, was used for the immunization. The titer of complement-independent antibodies against HSV-1 was 1:640, and that of complement-dependent antibodies was 1:1,280.

Complement. Freeze-dried pooled guinea pig serum was used as the source of complement. Undiluted serum contained 120 complement-fixing units.

Soluble HSV AM. LEP cells infected with HSV-1 (strain KOS) were used for preparing soluble antigen mixture (AM) (3). In brief, cells were disrupted by treatment with 0.5% Nonidet P-40 (Shell Chemical Co., Ltd., London, England) in isotonic reticulocyte standard buffer, pH 7.4. The extract was cleared of
Preparation of cells for CRT. The technique of Vasconcelos-Costa (13) was adopted, with minor modifications, for the $^{51}$Cr release cytotoxicity test (CRT). For all washings and dilutions, modified medium 199 (6) supplemented with 5% inactivated calf serum was used. RIF cells infected with HSV-1 (at a multiplicity of infection of about 0.2 plaque-forming units per ml) were dispersed by treatment with 0.2% trypsin in 0.02% ethylenediaminetetraacetate 22 h after infection. In different tests $3 \times 10^6$ to $20 \times 10^6$ washed cells were suspended in 0.3 ml of medium containing 25 to 150 µCi of $^{51}$Cr (as Na$_2$CrO$_4$, 106 Ci/g; Isocomertz, German Democratic Republic). After a 1-h incubation at 37°C with moderate shaking, the cells were washed four times with 7 ml of medium and finally resuspended at the desired concentration.

Preparation of cryopreserved target cells for CRT. Trypsinized HSV-infected and control noninfected cells were suspended in cryoprotective medium (maintenance medium without antibiotics containing 7.5% dimethyl sulfoxide) to a final concentration of $2 \times 10^6$ cells per ml. Ampoules with cells were frozen to $-25^\circ$C at a rate of 1°C per min in an alcohol-solid CO$_2$ mixture. The cells were then placed at $-70^\circ$C. Before being labeled with $^{51}$Cr, the cells were rapidly thawed in a 37°C water bath and washed with medium. The subsequent procedure was the same as that for the freshly infected cells.

CRT. Test samples for CRT consisted of 0.1 ml of medium, 0.1 ml of serum dilution, 0.1 ml of complement, and 0.2 ml of $^{51}$Cr-labeled cell suspension. The reaction mixtures, prepared in Eppendorf centrifugation tubes, were continuously shaken for 90 min at room temperature and then centrifuged for 15 s in an Eppendorf 3-200 centrifuge. Volumes of 0.2 ml of the supernatants were put into ampoules, and radioactivity was measured in a Packard gamma scintillation spectrometer for 5 min. Two types of control were included in each test. In the first, serum was replaced by medium to determine spontaneous chromium release. In the second, for maximum release determination, 0.8 ml of distilled water was added to 0.2 ml of target cells, and these mixtures were subjected to three cycles of freezing and thawing. After centrifugation, 0.4 ml of supernatant was taken for radioactivity determination.

The percentage of immune lysis was calculated by the formula proposed by Brunner et al. (1): percent specific $^{51}$Cr release = ($^{51}$Cr release in presence of serum and complement − spontaneous release)/(maximum release − spontaneous release) × 100.

CRIT. In the $^{51}$Cr release cytotoxicity inhibition test (CRIT), AM from HSV-infected cells was used instead of medium. Equal amounts (0.1 ml) of serum and serially diluted AM were incubated for 1 h at 37°C before the addition of complement and target cells. Thereafter, the test proceeded as described above. Inhibition of cell lysis was calculated according to Luborsky et al. (4): percent inhibition = [1− (percent $^{51}$Cr release in presence of antigen and serum/percent $^{51}$Cr release in presence of medium and serum)] × 100. The antigen titer was expressed as the reciprocal value of that AM dilution which caused a 50% inhibition of $^{51}$Cr release as compared with $^{51}$Cr release with antisera and complement alone.

RESULTS

Dependence of $^{51}$Cr release on complement, antibody, and cell concentrations. The effect of different factors on the percentage of specific $^{51}$Cr release was examined first. The

![Fig. 1. Dependence of $^{51}$Cr release on complement, antibody, and cell concentrations. Twofold dilutions of gamma globulin were tested in combination with 15 (△), 30 (○), 60 (□), or 120 (×) U of complement in mixtures with $4 \times 10^4$ (A) or $2 \times 10^5$ (B) HSV-1-infected cells. The highest activity in supernatants of test samples was about 3,600 cpm, maximum spontaneous release was about 1,900 cpm, and minimum spontaneous release was about 400 cpm.](http://iai.asm.org/)

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dependence of $^{51}$Cr release on complement and serum concentrations was studied in mixtures containing $4 \times 10^4$ or $2 \times 10^5$ target cells. The results of a representative experiment are shown in Fig. 1. They indicate that relative $^{51}$Cr release was higher with $4 \times 10^4$ than with $2 \times 10^5$ cells.

In both instances, the release was dependent on both antibody and complement concentrations.

**Conditions of CRIT.** In the next experiment, we studied the influence of different amounts of complement, antibody, and target cells on the sensitivity of the CRIT. A semilogarithmic plot

**Fig. 2.** Dependence of inhibition of $^{51}$Cr release on complement, antibody, and target cell concentrations. Different dilutions of AM-6 were reacted with various dilutions of gamma globulin and 15, 30, or 60 U of complement (c.u.) and $4 \times 10^4$ or $2 \times 10^5$ target cells. Only values of ≥10% inhibition of $^{51}$Cr release are indicated. Specific $^{51}$Cr releases for 200, 100, 50, 25, and 12.5 antibody units were, respectively: 49, 42, 28, and 19% (A); 72, 56, 47, and 30% (B); 88, 86, 76, and 47% (C); 25, 16, 12, and 6% (D); 45, 35, 24, and 13% (E); 78, 68, 58, and 32% (F).
of the reciprocal of AM dilution against the percentage of inhibition of \( ^{51}\text{Cr} \) release revealed a linear relation (Fig. 2). The highest titers of HSV antigens were found with the lowest concentrations of antibody and complement. The titers of HSV antigens were higher when using 2 \( \times 10^5 \) cells per tube than they were with 4 \( \times 10^4 \) cells per tube.

**Examination of the different AMs in CRT.** Six independently prepared AMs were examined in CRT. AM-1 was prepared from uninfected LEP cells; the other AMs were prepared from HSV-infected LEP cells as described elsewhere (3). The results are shown in Fig. 3. It can be seen that AM prepared from noninfected cells did not inhibit cytolysis. The other five AMs inhibited \( ^{51}\text{Cr} \) release at different rates. In all instances, the curves describing the relationship between inhibition of \( ^{51}\text{Cr} \) release and the reciprocal of antigen dilution were linear (in a semilogarithmic plot), and their slopes did not differ markedly. The content of HSV-specific antigens in the respective AMs as determined by the blocking neutralization test were <8 (AM-1), 96 (AM-2), 96 (AM-3), 96 (AM-4), 24 (AM-5), and 192 (AM-6).

**CRT and CRIT with cryopreserved target cells.** Big batches of serum (or gamma globulin) and complement, as well as a reference standard antigen preparation (used in every test), can be easily obtained for many repeated experiments. Target cells represent the most variable factor in the test. To reduce this variability we tried to use cryopreserved cells for both CRT and CRIT. \( ^{51}\text{Cr} \) release found with uninfected and HSV-1-infected cells at two durations of storage at \(-70^\circ\text{C}\) is shown in Table 1. In this test, rabbit HSV-1-hyperimmune serum diluted 1:10, 120 U of complement, and 2 \( \times 10^5 \) cells per tube were used. It is apparent that frozen cells were still fully acceptable for CRT after 3 months of storage.

Figure 4 shows results of CRIT with freshly infected cells and cells which had been kept for 3 months at \(-70^\circ\text{C}\). The results are clearly comparable.

**DISCUSSION**

Numerous studies (e.g., 8, 11, 14) have furnished evidence that the viral antigens present on the surfaces of HSV-infected cells are the same as or similar to those of the virion surface. This information forms the basis for the quantitation of antibodies to HSV in the \( ^{51}\text{Cr} \) release test (5, 11) and also for the titration of solubilized HSV antigens in CRIT, as has been described in this work.

The data presented above show that the titer of HSV antigens was dependent on complement, antibody, and cell concentrations. The highest titer was found in samples containing the least

![Graph showing percent inhibition of \( ^{51}\text{Cr} \) release against AM dilution.](image)

**FIG. 3.** Comparison of six independently prepared AMs in CRT. Gamma globulin diluted 1:80, 30 U of complement, and 4 \( \times 10^4 \) cells were used in the reactions. Specific \( ^{51}\text{Cr} \) release in different control samples without AMs was 55 to 69%. Symbols: ■, AM-1 (prepared from uninfected cells); △, AM-2; ○, AM-3; ◊, AM-4; ◇, AM-5; ●, AM-6 (latter five prepared from HSV-infected cells).

<table>
<thead>
<tr>
<th>Storage time at (-70^\circ\text{C}) (weeks)</th>
<th>( ^{51}\text{Cr} ) release (%)</th>
</tr>
</thead>
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<tr>
<td>Uninfected cells</td>
<td>HSV-1-infected cells</td>
</tr>
<tr>
<td>0</td>
<td>87.6</td>
</tr>
<tr>
<td>4</td>
<td>80.6</td>
</tr>
<tr>
<td>12</td>
<td>86.9</td>
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**TABLE 1.** \( ^{51}\text{Cr} \) release test with cryopreserved cells

![Graph showing percent inhibition of \( ^{51}\text{Cr} \) release against dilution of AM-6.](image)

**FIG. 4.** Comparison of freshly infected and cryopreserved infected cells in CRT. Different dilutions of AM-6, 30 U of complement, Gamma globulin (GG) diluted 1:160 and 1:80, and 4 \( \times 10^4 \) cells per tube were used. \( ^{51}\text{Cr} \) releases in control samples without AMs were 22% (○, GG 1:160, cryopreserved infected cells), 26% (●, GG 1:160, freshly infected cells), 44% (△, GG 1:80, cryopreserved infected cells), 52% (■, GG 1:80, freshly infected cells).
amounts of complement and antibodies; with increasing concentrations of both components, the antigen titer decreased. However, the use of a low complement concentration is not suitable for routine work because of the low ^51Cr release from which the inhibition effect must be calculated. We therefore suggest that 30 U of complement, about 20 complement-dependent neutralizing antibody units (as determined by the present techniques), and 4 × 10^7 target cells constitute the optimal conditions for CRIT. The rather low concentration of cells should be given preference for economic reasons and because the relative ^51Cr release is higher than in samples with 2 × 10^6 cells.

When several independently prepared AMs were tested in CRIT, in semilogarithmic plots it was found that the relationship between inhibition of ^51Cr release and the reciprocal of AM dilution was linear and that all the lines had similar slopes. Thus, theoretically, the measurement of inhibition of ^51Cr release in two dilutions of a given AM should be sufficient for determination of the HSV antigen content in the AM. On the basis of the correlation obtained between the results of CRIT and the blocking neutralization test, we assume that both tests measure the same activity, i.e., HSV neutralizing antigens.

In results similar to those reported by Thong et al. (12) for cytomegalovirus-infected cells, in our hands HSV-1-infected cells kept frozen at −70°C and freshly infected cells gave comparable ^51Cr releases. A similar high degree of stability was found for frozen cells infected with HSV-2 (data not given). The use of cryopreserved cells in CRT and CRIT seems to facilitate high-level reproducibility in different experiments.

Taking into consideration the simplicity, reproducibility and sensitivity of CRIT, this test may be expected to prove useful not only in studies on HSV but also in studies of other enveloped viruses.

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