A Fluoroimmunoassay for Measurement and Visualization of Antibody Bound to Surface Antigens of HSV-Infected Cells

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SUMMARY

An assay based on quantitative spectrofluorometry of surface immunofluorescence was applied to the study of reactions between antibody and surface antigens of viable HSV-infected cells in suspension. Fluorescence was expressed in terms of photon counts per second. Both the direct and indirect fluorescent antibody techniques proved acceptable for assay of surface reactions, yielding values of specific fluorescence as high as eight times those of controls. Fluorescence microscopy of cell populations assayed by spectrofluorometry allowed for simultaneous visual examination of surface antigens.

Comparison of the fluoroimmunoassay with the 51Cr-release test for cytolytic antibody to HSV-induced surface antigens revealed the latter to be the more sensitive, with antibody titres ranging up to four times those detected by fluoroimmunoassay. General correlation between the two assays was found using both rabbit and human sources of antisera.

INTRODUCTION

Interactions between antibody and surface antigens of cells infected with herpes simplex virus (HSV) appear to be of major importance in host control of herpetic infection (Hilleman, 1976; Watson & Honess, 1977). Rapid progress has been made in determining the characteristics of antibody and antibody-dependent effector cells reactive with the surface of the infected cell. Progress in characterizing the antigens involved in these reactions, however, has lagged behind.

Three general types of assay are currently in use to analyse interactions between antibody and viable cells infected with HSV. These are: (1) direct binding by surface antigens of antibody labelled with 125I, ferritin, or fluorescein (Nii et al. 1968; Smith et al. 1972b; Hayashi et al. 1973/4); (2) assays for complement-mediated cytolysis using 51Cr-release or trypan blue exclusion as indices (Smith et al. 1972a); and (3) assays in which antibody to virus is used to identify virus-coded proteins in the membranes of cells that have been surface-labelled with 125I (Glorioso & Smith, 1977). Only with assays in the first category, however, which involve direct binding of antibody to intact cells, can the distribution of reactive sites on the cell-surface be discerned.

Methods employing fluorescein-conjugated antibody have had the widest application in detecting antigen-antibody reactions in many biosystems. Fluorescence microscopy as
conventionally applied, however, is only semi-quantitative at best (Cherry, 1974). In recent studies, fluorescence of particles in suspension (Burgett et al. 1977), including bacteria (Gillis & Thompson, 1978), was measured by fluorimmunoassay (FIA). By this assay a quantitative relationship is established between the antigen–antibody reaction and the intensity of fluorescence per particle. We used FIA to analyse quantitatively the reaction of antibody to HSV with viable HSV-infected cells in suspension and correlated the results with those observed by conventional fluorescence microscopy.

**METHODS**

*Antisera.* Fluorescein-conjugated rabbit antibody to HSV-1 was purchased from Flow Laboratories, Rockville, Md., U.S.A. Antiserum was also prepared in rabbits by immunization with partially purified HSV-1 (strain KOS) in complete Freund’s adjuvant as previously reported (Smith & Glorioso, 1976). Fluorescein isothiocyanate-conjugated goat-anti-rabbit IgG (FGAR-IgG) was purchased from Cappel Laboratories, Cochranville, Pa., U.S.A.

*Infection of cells.* BHK-21 cells were grown to confluency in 16-oz prescription bottles in Eagle’s minimal essential medium with 10% foetal calf serum (MEM-FCS), streptomycin (100 μg/ml) and penicillin (100 units/ml). Monolayers were infected with HSV-1, strain KOS, at a m.o.i. of approx. 10 p.f.u./cell and incubated at 37 °C for 20 h.

*Reaction of cells with antisera.* Monolayers were dispersed with EDTA (0.5 mM) and washed three times by alternate centrifugation (200 g for 10 min) and resuspension in 10 ml amounts of MEM-FCS. More than 90% of the cells remained viable, as determined by trypan blue exclusion. We then added 100 μl of antiserum to 2 x 10^6 cells in 100 μl of phosphate-buffered saline (PBS), pH 7.5, in 12 x 75 plastic tubes (Falcon Plastics, Oxnard, Calif., U.S.A.). After incubation for 45 min at 37 °C, the cells were washed three times in PBS to remove unreacted antibody. In direct tests, after reaction with labelled antibody, the cells were resuspended in 500 μl PBS for measurement of fluorescence. In indirect tests, after reaction with primary antibody to HSV-1 for 45 min at 37 °C, the cells were resuspended in FGAR-IgG (100 μl), incubated for 45 min at 37 °C, washed three times in PBS and resuspended in 500 μl of PBS for measurement of fluorescence.

*Measurement of fluorescence.* Fluorescence was determined by modification of a method previously described (Gillis & Thompson, 1978). To measure fluorescence, a 0.3 ml quartz cuvette assembly (Farrand Optical, Valhalla, N.Y., U.S.A.) containing 300 μl of cell suspension was placed in an MKI spectrophotometer (Farrand Optical) modified for photon counting by the addition of a Model 1140 quantum photometer (Princeton Applied Research Corp., Princeton, N.J., U.S.A.). Readings were taken 1 min after insertion of the cuvette and required about 5 s to complete. Fluorescence was expressed in terms of the photon counting rate in counts per second (ct/s) at a standard deviation of 1.3% for full scale readings. Crossed film polarizers were used to minimize interference from light scatter (Chen, 1966). Slits were chosen to allow a 10 nm bandpass. The excitation monochromator was set at 485 nm and the analyser monochromator set at 525 nm. To determine specific activity, counts obtained from reaction of test sera with uninfected cells were subtracted from counts obtained using infected cells.

In earlier studies with a bacterial system (Gillis & Thompson, 1978), we demonstrated that loss of fluorescence due to settling of bacteria in suspension was not significant. Because of their size, fluorescein-labelled BHK-21 cells tended to settle in the cuvette. As determined by kinetic studies of fluorescent cells during a 30 min interval, this settling resulted in a decrease of fluorescence intensity of less than 2% per min. Because of the short period
required to measure fluorescence (5 s), we found that the error introduced by settling was so small as to be indistinguishable from experimental errors introduced by other manipulations, such as pipetting and washing.

**Visualization of surface antigens.** For direct visualization of surface antigens, fluorescent cell pellets (2 × 10⁶ cells), derived by either the direct or indirect techniques described above, were resuspended in 100 µl of PBS on a glass slide. Coverslips were placed over the suspensions and the edges sealed with nail polish. Fluorescence was observed with a Reichert Zetopan microscope equipped with incident light illumination (Reichert, Vienna, Austria). Alternatively, fluorescent cells on slides were air dried and fixed in methanol before coverslips were added (Smith et al. 1972b).

**Cytolytic antibody assay.** Cytolytic antibody to surface antigens of HSV-infected cells was measured by a modification of the ⁵¹Cr-release test previously described (Smith et al. 1972a). Cells were infected as described above and incubated at 37 °C. After 8 h, 200 µCi of ⁵¹Cr (sodium chromate; Amersham-Searle, Arlington Heights, Ill., U.S.A.) was added to each monolayer and the cultures were incubated for an additional 12 h. Monolayers were dispersed into single cells and divided into amounts of 2 × 10⁶ in 100 µl of PBS as described for the fluoroimmunoassay. One hundred µl of antisera diluted in PBS was then added in triplicate and the resulting mixtures incubated for 45 min at 37 °C; 100 µl of guinea pig serum (complement source), diluted 1:5, was added to each tube, bringing the total vol. to 300 µl. After 1½ h of incubation at 37 °C, 1 ml of cold PBS was added and the cells were pelleted by centrifugation at 400 g for 10 min. One ml of supernatant fluid was removed from each tube and monitored for ⁵¹Cr-release activity in a Beckman Biogamma scintillation counter. Controls for each assay consisted of tubes containing only complement and ⁵¹Cr-labelled cells (spontaneous release) and tubes containing labelled cells that had been frozen and thawed twice (maximum release). Cytolytic antibody activity was expressed as percentage specific ⁵¹Cr-release according to the formula:

\[
\% \text{ specific } ⁵¹\text{Cr-release} = \left( \frac{\text{³¹Cr in presence of test} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \right) \times 100.
\]

**RESULTS**

**Measurement of fluorescence**

For the indirect technique, serial twofold dilutions of unlabelled rabbit antibody to HSV-1 were reacted with viable HSV-infected cells. To each primary reaction mixture, FGAR-IgG was added in a dilution (1:10) representing antibody excess, as determined by separately performed titrations. As shown in Fig. 1(a), at the highest concentration of primary antibody (1:5), specific fluorescence was at least eight times that of controls. The counts decreased with increasing dilutions of antisera, indicating that fluorescence was dependent on the concentration of specific antibody attached to the cell surfaces.

In the direct technique (Fig. 1b), performed under the same conditions, a similar decline in fluorescence was observed with increasing dilution of fluorescein-labelled antibody to HSV-1. The difference, not unexpectedly, between specific and control counts was not as high when the direct technique was used. These observations agree with those of other investigators who demonstrated the greater sensitivity of indirect techniques for detection of HSV-induced surface antigens (Espmark et al. 1971).
Fig. 1. Measurement of surface fluorescence of HSV-infected cells after reaction with antibody. (a) Indirect technique: △—△, antibody to HSV reacted with HSV-infected cells; □—□, pre-immune serum reacted with HSV-infected cells; ●—●, antibody to HSV reacted with uninfected cells; ○—○, pre-immune serum reacted with HSV-infected cells. (b) Direct technique: ○—○, fluorescein-conjugated antibody to HSV reacted with HSV-infected cells; ●—●, fluorescein-conjugated antibody to HSV reacted with uninfected cells.

Visualization of fluorescence

Fig. 2 shows the patterns and relative intensity of fluorescence of antibody bound to surface antigens of viable cells. The exposure time was the same for all micrographs to bring out quantitative differences among the several preparations. Uninfected cells reacted with a 1:5 dilution of primary antiserum to HSV gave counts of 3.4 × 10⁴ ct/s (Fig. 1a) and showed little visible fluorescence (Fig. 2d). Infected cells that were reacted with the same dilution of virus antibody gave counts exceeding 3.25 × 10⁵ ct/s and were brightly fluorescent with FGAR-IgG (Fig. 2a). Infected cells reacted with a 1:5 dilution of fluorescein-conjugated antiserum to HSV in the direct technique were intermediate in intensity (Fig. 2b), corresponding to the intermediate number of counts obtained (1.9 × 10⁵ ct/s, Fig. 1b).

The most common pattern of fluorescence observed on infected cells is shown in Fig. 2(d). The entire cell surface fluoresced with even intensity, slightly accentuated by lines of increased fluorescence probably representing folds in the cell membrane. These observations suggest that the virus antigens were evenly distributed over the cell surface and that capping and endocytosis did not occur to any significant extent.

Direct counts revealed that 100% of the cells were infected. By dividing ct/s by the number of cells present in the counting cuvette (1.2 × 10⁶ cells in 0.3 ml), a value representing the average minimum fluorescence per cell can be obtained. Under the test conditions represented in Fig. 2(a) (3.3 × 10⁶ ct/s), for example, the value is 2.8 ct/cell. By this technique surface fluorescence can be expressed in terms of single cell units. This capability should facilitate comparison of different cell populations. In addition, preliminary experi-
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Fig. 2. Micrographs of representative cell suspension from Fig. 1. (a) HSV-infected cells reacted with a 1:5 dilution of antibody to HSV, followed by FGAR-IgG (indirect technique). (b) HSV-infected cells reacted with a 1:5 dilution of conjugated antibody to HSV (direct technique). (c) Uninfected cells and a 1:5 dilution of antibody to HSV, followed by FGAR-IgG (indirect technique). (d) Characteristic pattern of surface fluorescence (2.5 times magnification of a, b and c).

Experiments not detailed here have shown that solubilization of labelled HSV-infected cells with 0.5% deoxycholate has no effect on immune-specific fluorescence. Thus, intact cells may not be necessary for measurement of surface fluorescence, a finding which has been recently verified in studies on quantification of Fc receptors by fluoroimmunoassay (Schreiber et al. 1978).

Comparison of fluoroimmunoassay with 51Cr-release assay for cytolytic antibody

An assay which has been commonly used to measure interactions between antibody and virus-induced surface antigens is the 51Cr-release test for complement-dependent cytolytic antibody. Comparisons were made between this assay and the fluoroimmunoassay for detection of antibody to surface antigens. Results of examination of four antisera produced in rabbits, two against HSV-1 and two against HSV-2, are shown in Fig. 3. In order to minimize experimental error, the assays were run in parallel using the same source of infected cells. Based on extinction of sp. act., the 51Cr-release assay was found to be about twofold more sensitive than the fluoroimmunoassay. Considerable variation was noted at highest concentrations of antibody, suggesting that titration of an antisera is required to obtain clear relationships between the two assays. This variation was particularly evident with the antisera produced against HSV-2 (Fig. 3b, d).
Fig. 3. Antibody to surface antigens of HSV-infected cells detected by fluoroimmunoassay (●—●) and the $^{51}$Cr-release test (○—○). Antisera were reacted with cells infected with HSV-1. Points represent sp. act. (a) and (c) Antisera prepared in rabbits against HSV-1. (b) and (d) Anti- sera prepared in rabbits against HSV-2.

Table 1. Antibody to HSV-induced surface antigens measured by fluoroimmunoassay and the $^{51}$Cr-release test

<table>
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<tr>
<th>Antisera examined</th>
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<tr>
<td></td>
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*Antisera were diluted in twofold steps and reacted with cells infected with HSV-1. Values represent the reciprocal of the first dilution at which sp. act. was no longer detected.
† Negative for antibody to HSV-1 by the microneutralization assay (Rawls et al. 1970).
‡ Classified as positive for exposure to HSV-1 on basis of presence of type-specific antibody to HSV-1 with no evidence of specific antibody to HSV-2 (Smith & Glorioso, 1976).
Results from the examination of an additional thirteen sera, eight rabbit and five human, are shown in Table 1. Although examination of a few of these sera revealed the same titration endpoints, the $^{51}\text{Cr}$-release test was again found to be more sensitive in most cases in this larger sampling. As shown by the low titres found in sera obtained from serologically negative humans and normal rabbits, there was general correlation between the two assays. The endpoints determined from the two assays appeared to be unaffected by the source of antiserum, antigenic type of HSV used for immunization (rabbits), or the clinical history of herpetic disease (humans).

**DISCUSSION**

Other approaches to quantitative FIA of cell suspensions have been reported. The procedure described by Strom & Klein (1969), using a conventional filter fluorometer, proved to be relatively insensitive, required large numbers of cells, took several days to complete and required solubilizing the cells before fluorescence could be measured. As a result of these drawbacks, microspectrofluorometry supplanted that earlier technology (Espmark et al. 1971; Capel, 1974). However, microspectrofluorometry requires that fluorescence of many individual cells be measured in order to arrive at a value representing the mean intensity fluorescence per cell of a given population. Similar limitations hold in interpretations of data derived from flow microfluorometry (Bailey et al. 1977).

In contrast, the fluoroimmunoassay method we describe here permits direct measurement of the average fluorescence of a predetermined number of cells in suspension. Examining a large number of individual cells is unnecessary and the need for computer weighting and averaging of pulse height distributions from flow fluorometry data is avoided. Additional advantages of our FIA methods over microspectrofluorometry and flow fluorometry include: (1) the short time required for completion of an assay (5 s for a reading after a 1 min settling period and a few hours for incubation and washing); (2) the visualization, as well as measurement, of the reaction of antibody with cell surface antigens in a single preparation; and (3) the quantitative expression of fluorescence in units (ct/s) amenable to standardization by accepted experimental methods.

Our results indicate that fluoroimmunoassay is less sensitive than the $^{51}\text{Cr}$-release test in most cases for detecting antibody to surface antigens. The degree of difference in sensitivity varied with the serum samples examined, however, probably reflecting differences in antibody populations and specificities for particular surface structures. The differences incurred are not surprising, since the two assays measure different kinds of interaction with infected cell surfaces. The fluoroimmunoassay measures binding without reference to function, while the $^{51}\text{Cr}$-release test detects only those immunoglobulins whose interactions with surface antigens leads to cytolysis. The utility of fluoroimmunoassay with regard to the $^{51}\text{Cr}$-release test, however, lies in its ability to quantify surface antigens, a procedure which now usually requires preparations of radiolabelled antibody.

In summary, we have found that interactions between antibody and surface antigens of virus-infected cells can be studied directly by fluoroimmunoassay. With appropriately standardized primary antisera and fluorescein-conjugated antiglobulin reagents, absolute quantification of immune reactions may be feasible. However, even in the absence of standardized reagents, the method is sufficiently rapid and sensitive that relative measurements of antibody bound to surface antigens may be rapidly obtained on large numbers of samples.

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REFERENCES


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