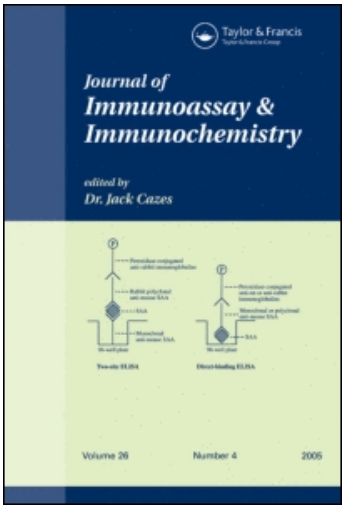

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Quantitative Determination and Visualization of Herpes Simplex Virus Type 1 Antigen, Free and Cell-Bound by Elisa.

Rocío Tirado[a]; Rosa Elena Sarmiento[a]; Beatríz Gómez[a]
[a] Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, México

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QUANTITATIVE DETERMINATION AND VISUALIZATION OF HERPES SIMPLEX VIRUS TYPE 1 ANTIGEN, FREE AND CELL-BOUND BY ELISA.

Rocío Tirado, Rosa Elena Sarmiento, and Beatríz Gómez*
Universidad Nacional Autónoma de México, Departamento de Microbiología y Parasitología, Facultad de Medicina, México, D.F. 04510

ABSTRACT

A method of quantifying and visualizing herpes simplex virus type 1 antigen by indirect enzyme-linked immunosorbent assay (ELISA) is described. This assay is simplified by the use of polyclonal serum and can be applied to the quantification of free antigen as well cell-bound. Moreover, cell viral antigen can be visualized. Antigen sources were viral suspensions, infected cells and proteins extracted from infected cells. The assay was specific and its sensitivity was dependent on the antigen source. The technique was regarded as specific within a range showing a direct correlation ($r>0.8$) between the concentration of the antigen and the net absorbance value (the difference of the absorbance obtained with the viral antigen minus the control antigen). The technique has advantages over other ELISA procedures: does not require monoclonal antibodies, or labelled antiviral immunoglobulins or antiviral serum from two different species. In addition total free antigen can be measured.

KEY WORDS: ELISA/Antigen HSV free and cell-bound)

INTRODUCTION

Traditional techniques used to detect and quantify viral antigens such as hemagglutination, electron microscopy,

*Author to whom correspondence should be sent

immunoflurescence and viral infectivity have been substituted by the immunosorbent enzyme-linked assay (ELISA) [1]. This procedure take advantage of the specificity of the antibody-antigen reaction, the use of enzymes as indicators to detect and quantify the antigen-antibody interaction and the amplification of chemical reactions with enzymes. Moreover, the specificity of the antibody to a given antigen is conserved when one of the components is absorbed in a solid phase. The procedure is easy to perform, reproducible, highly sensitive and specific. Furthermore, the reagents have a long shelf-life; the results can be obtained in a few hours and the health hazards are limited, since viral infectivity is not required [1,2].

Although ELISA assays have been used for detecting and quantifying herpes simplex virus type 1 (HSV) antigen in suspension, the reported techniques have some limitations for routine work in laboratories with limited resources: a sensitive and specific assay requires either monoclonal antibody [indirect ELISA; 3,4,5] or anti-viral immunoglobulin from two different species (sandwich ELISA [6]) or labeled anti-HSV (direct ELISA) [7]. Moreover, the reported techniques use commercial anti-HSV antibody [5] or kits [3,7,8,9], increasing the cost of the assay. Furthermore, detection, quantification and visualization of HSV antigen in infected cells has not been reported.

In our laboratory we routinely determine and quantify free HSV antigen by viral infectivity, which, although sensitive and specific, has some drawbacks: the infectivity is labile, it does not detect non-infectious free antigen, requires cell cultures and the results are obtained after several days.

With the aim of having in the laboratory a rapid, specific and sensitive assay to be used as a routine technique for: 1) detecting and quantifying free and cell-bound HSV antigen and 2)visualizing cell-viral antigen, we decided to adapt the indirect ELISA procedure employing polyclonal serum as a source of antiviral antibody. Anti-HSV serum is frequently used in the laboratory and therefore it is readily available.

MATERIALS AND METHODS

VIRUS AND CELLS

The macro plaque strain of HSV was used, kindly provided by Dr. Bernard Roizman (University of Chicago, Chicago, ILL.). Vero cells(African Green Monkey kidney) and HEp-2 (Human, epidermoid carcinoma, larynx) originally from American Type Culture Collection (CCL-81 and CCL-23) were used and maintained in minimal essential medium (Gibco LBR), supplemented with antibiotics (100 units penicillin/ml; 100 ug/ml streptomycin, Sigma Chemical Co.), hereafter called MEM, to which was added 5% of heat-inactivated (56°C for 30 min) newborn calf serum (Bioexport). Viral stocks were obtained in Vero or HEp-2 cells, viral infectivity was determined in the same cell line as viral propagation and its titer expressed in plaque forming units per milliliter (pfu/ml) [10].

ANTI-HSV SERUM

Antiviral serum was obtained from a New Zealand rabbit (2.5-3.0 Kg) immunized with three intracutaneous injections at intervals of 21 days. A volume of 0.5 to 1 ml of virus suspension obtain in HEp-2 cells with a titer of $2X10^4$ pfu/ml in MEM and a protein content from 200 to 300 ug/ml determined by Lowry [11] was used in each injection. Complete Freund´s adjuvant (1:1 vol/vol; Sigma) was used in the first injection and incomplete adjuvant in the others. Serum was obtained 8 days after the last immunization. Serum used in the ELISA assay was absorbed three times with Vero cells: to 1 ml of serum were added 10^5 cells, incubated with agitation for 1h at 37°C, centrifuged at 1,500 rpm for 10 min. The supernatant was collected and stored at -20°C until use. Normal serum was obtained from a non-immunized rabbit and was treated as anti-HSV.

NEUTRALIZATION ANTIBODIES

The concentration of neutralization antibodies in the serum was determined by inhibition of plaque formation and after heat inactivation at 56°C for 30 min. To 50 ul of the concentrated rabbit serum and to serially diluted serum (1:3 in MEM) were added 50 ul of the viral stock obtained in HEp-2 cells with titers from 10^5 to 10^6 pfu/ml, incubated for 1 h at 37°C and viral infectivity was determined. Titers were expressed in neutralized pfu/ml, they varied between $1X10^5$ and $5X10^5$.

VIRAL ANTIGENS

Viral stocks obtained in Vero and diluted in MEM at concentrations between 5 and $4X10^4$ pfu/ml (viral suspensions) were used as free antigen. Cell-bound antigens were determined or visualized in Vero infected cells, either in the cells attached to the well of a 96 well plate (Costar) or after extraction of the proteins (protein extract).

To determine or visualize cell-bound antigen in attached cells, each well was coated with 10^4 Vero cells and infected at a multiplicity of infection (virus/cells; moi) ranging from $2X10^{-6}$ to $2X10^{-1}$. After incubation for 24 or 48 h, the wells were washed with PBS and fixed with 5% of H_2O_2 in methanol for 45 min at room temperature. Then they were washed three times with 0.5% (w/v) of bovine serum albumin (Sigma) in PBS (PBSA) and viral antigen was determined by ELISA.

Protein extracts were prepared from 10^6-10^7 Vero cells grown in Petri dishes (Costar) and infected at moi from 10^{-4} to 10^1. When a clear cytopathic effect was observed (24 to 96 h post-infection; pi), the cells were scraped with cell scrapers (Costar), centrifuged at 1,500 rpm for 10 min, washed three times with PBS, centrifuged again and the pellet was suspended in 2 ml of lysis buffer (10 mM Tris-HCl pH 7.5, 140 mM NaCl, 5 mM EDTA, 1% triton X100, 1% Na Deoxicholate (Sigma). They were vortexed for 30 sec and centrifuged at 1,500 rpm for 5 min at room temperature.

The sediments were collected, protein contents determined [11] and adjusted with PBS to 1.7 mg/ml.

Free antigen or viral antigen in protein extracts were absorbed to ELISA polystyrene microplates (Inmulon II Dynathech). 100 ul of either viral suspensions or protein extract were added to each well. After overnight absorption at 4°C, the plates were washed with PBSA +0.05% Tween 20 (Sigma; PBST); thereafter viral antigen was determined by ELISA.

Control antigens were obtained from mock-infected cells using the same procedures.

ELISA ASSAY

The antigen absorbed to the ELISA plate or in the fixed cells was determined after blocking by adding to each well 100 ul of PBSA and incubation for 90 min at 37°C. After washing with PBST, 200 ul of anti-HSV serum, diluted in PBSA (1:100) were added and incubated for 1h at 37°C. After washing with PBST, 100 ul of donkey anti-rabbit IgG, conjugated to peroxidase (1:1000; Amersham Life Sciences), was added. After the plates had been incubated (1 h, 37°C) and washed three times with PBST, substrate (100 ul/well) was added. When the peroxidase activity was measured by spectophotometry, 0.04% o-phenylendiamine (Sigma) in 1 M disodium citrate buffer (pH 5.0) and 0.05% H_2O_2 was used as substrate and the absorbance (OD) was measured at 492 nm in an ELISA reader. The substrate used for visualizing the antigen in the infected cells was 5% 3-3´ diaminobenzidine (Sigma) in 0.5 M Tris-HCl buffer (pH 7.6) with 0.05% H_2O_2. The enzyme reaction was left for 20-30 min and stopped with 50 ul/well of either 3 M H_2SO_4 or 10% formaldehyde in PBS and the plate observed with an inverted microscope. The presence of a brown precipitate was considered as positive

SENSITIVITY AND SPECIFICITY.

The sensitivity limit of the technique was defined by the cut-off line, its value was calculated for each series of samples and

every assay was done in triplicate. The cut-off value was the mean of the absorbance (OD) given by the viral antigen with normal serum plus three standard deviations [12], OD values between 0.04 and 0.06 were obtained. Normal serum with control antigen yielded OD readings of 0.02 to 0.035.

Any assays with net absorbance value above the cut-of line were considered specific. Net absorbance was calculated according to the formula:
(mean of the OD of viral antigen with anti-HSV serum minus cut-of value)-(mean of the OD of control antigen with anti-HSV serum minus cut-of value).

The specificity of the assay was defined by the direct correlation between the net absorbance value and the antigen concentration. Curves were obtained by plotting the net absorbance value of each sample versus antigen concentration. Antigen concentration for free antigen was expressed in form pfu/ml and for cell bound in moi.

STATISTICS

The correlation coefficient (r) between the antigen concentration and the net absorbance values were calculated with 95% of confidence and by linear-regression. All calculations were performed using a personal computer and EPIINFO software [13].

RESULTS

QUANTIFICATION OF FREE ANTIGEN

Initially, when polyclonal serum was used without pretreatment with cells, the OD values obtained with viral and control antigen were practically the same. Moreover, an increase in the antigen concentrations was not reflected in an increase of the net absorbance values. Therefore the assay was considered unspecific.

However, by absorbing the sera with Vero or HEp-2 cells, net absorbance values over the cut-off line were observed. Treatment

with Vero cells yielded better results than with HEp-2, therefore Vero cells were used.

Assays at antigen concentration between $1.5X10^{2}$ and $4X10^{4}$ pfu/ml, and with treated sera were specific. An increase in the antigen concentration was reflected in the net absorbance value. Evidence of specificity was the coefficient of correlation value between net absorbance readings and antigen concentration. The results are shown in Fig. 1A and 1B.

As seen in Fig.1A, the response of the ELISA reaction to the antigen concentration is non-linear. To show the values of low antigen concentrations more clearly, the curves were plotted on a logarithmic abscissa and the cut-off values taken as zero (see Fig.1B). In order to use curves such as that seen in Fig. 1B, it is important that the boundaries of specific response be defined (see Methods). In the experiment of Fig. 1A and 1B the boundaries of specificity were defined to be from $1.5X10^{2}$ to $4X10^{4}$ pfu/ml, in that range the correlation coefficient was of r=0.8.

QUANTIFICATION OF CELL-BOUND ANTIGEN IN ATTACHED CELLS

Cell-bound antigen in attached cells was determined by spectrophotometry and by immunocytochemistry. Fig. 2A and 2B illustrate the results obtained when antibody-antigen complexes were measured by spectophotometry at moi from $2X10^{-6}$ to $3X10^{-1}$ and at 24 (A) and at 48 (B) h pi.

Altough the net absorbance values in assays performed at 24 and 48 h pi were above the cut-off line, an increase in the antigen concentration did not significantly increase the net absorbance value when moi was less than $8X10^{-5}$ and after 24 h of infection. The correlation coefficient between these parameters and at moi from $8X10^{-6}$ to $8X10^{-2}$ was not significant ($r<0.8$). Therefore, in this range the technique was not reliable.

Nonetheless, the test was specific at antigen concentrations (moi, $2X10^{-5}$ to $8X10^{-2}$) when the time after infection was kept constant at 24 h pi (r=0.95). After prolongation of the time to 48 h pi, the proportionality of the test and its sensitivity to

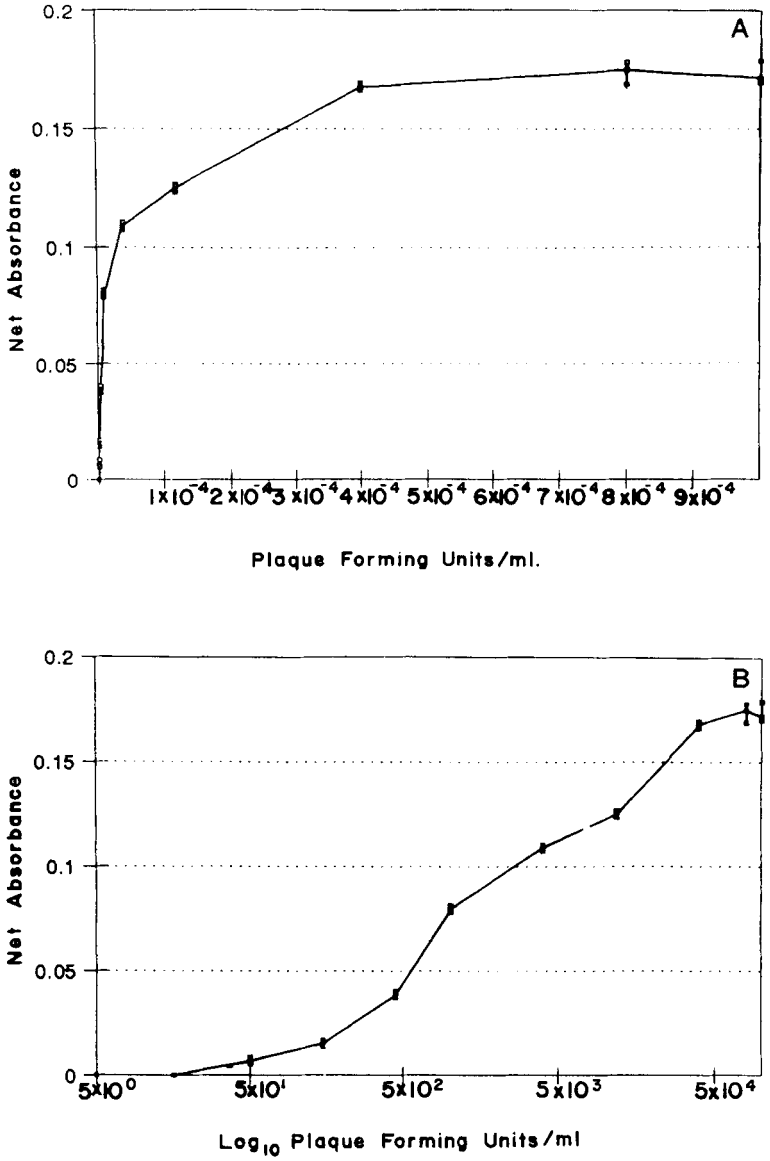

FIGURE 1. Quantification of HSV free antigen. The plotted net absorbance values are the mean of three assays. The cut-off value of 0.04 OD was taken as zero. Data points under $1.5X10^2$ and over $4X10^4$ pfu/ml were omitted from the linear regression analysis since they were under the boundary of specific response. The correlation coefficient value was of 0.8.

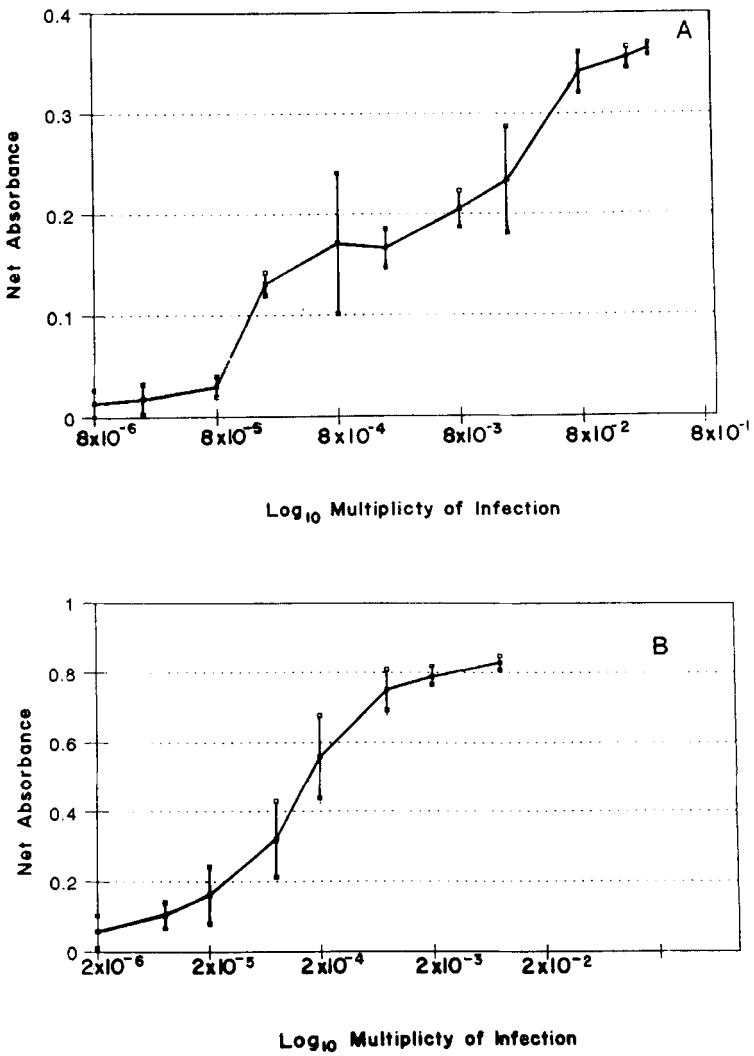

FIGURE 2. Quantification of HSV cell-bound antigen in attached cells. The plotted net absorbance values are the mean of three assays. The cut-off value of 0.04 OD (A) and 0.05 OD (B) was taken as zero. (A) After 24 h pi, data points under multiplicity of infection of $8X10^{-4}$ and over $8X10^{-2}$ were omitted from the linear regression analysis since they were under the boundary of specific response. (B) After 48 h pi data points over $8X10^{-4}$ were omitted from the linear regression analysis. The correlation coefficient values for (A) and (B) were of 0.95 and 0.8 respectively.

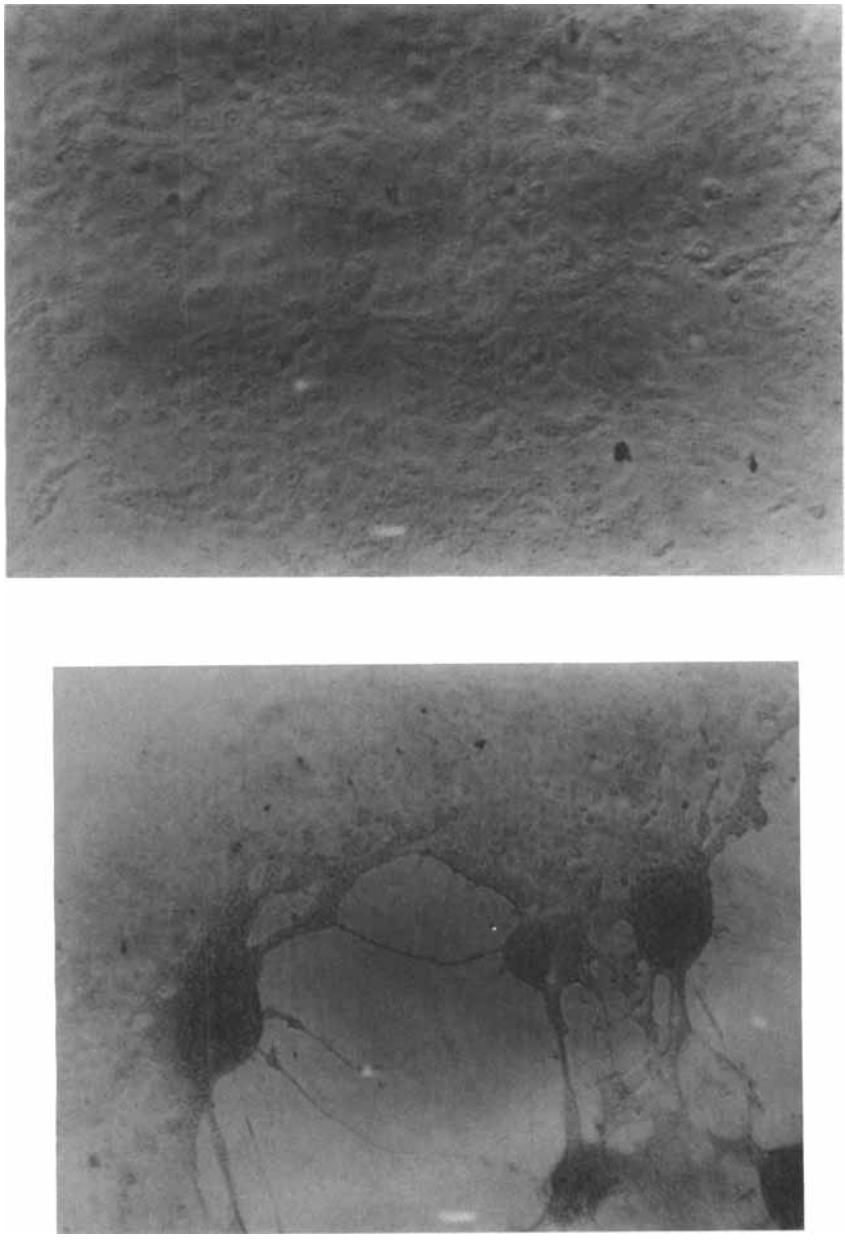

FIGURE 3. Visualization of cell-bound HSV antigen in Vero cells by indirect immunoperoxidase. (A) Non-infected cells; (B) Infected cells at moi of $1X10^{-2}$ and after 48 h of infection.

higher antigen concentrations ($>8X10^{-4}$) is lost. This is illustrated by the curve of Fig. 2B which tends to level off at high concentrations. Nonetheless, at 48 h pi and at lower antigen concentrations (moi $2X10^{-6}$ to $8X10^{-4}$) a correlation coefficient value of r=0.8 between the antigen concentration and the net absorbance value was obtained, implying that the assay was specific.

Even though in the immunochemical assay viral antigen could be visualized at 24 h and at 48 h pi, the color of the precipitate was more intense at 48 h pi Therefore we decided to continue work with this time lapse. Fig. 3 shows HSV antigen in Vero cells at 48 h after infection.

QUANTIFICATION OF CELL-BOUND ANTIGEN IN PROTEINS EXTRACTED FROM INFECTED CELLS

Results obtained in the quantification of viral antigen in proteins extracted from infected cells at moi from $1X10^{-4}$ to $2X10^{1}$ are shown in Fig. 4. The Figure shows that in all the assays the net absorbance values are above the cut-off line and by increasing the antigen concentration an increase in the net absorbance values was observed. However, no correlation between net absorbance and antigen concentration was observed in assays done at moi higher than $1X10^{1}$. The technique was specific in the antigen concentration limits of moi $1X10^{-4}$ to $1X10^{1}$, the correlation coefficient value on that range was of r=0.97.

DISCUSSION

The indirect ELISA systems reported here are specific methods to quantify free and cell-bound HSV antigen with certain limits. Our interest in developing an ELISA assay of free and cell-bound HSV antigen is based on the simplicity of the technique and polyclonal serum can be use. It does not rely on the replication capacity of the virus, and therefore it does not depend on its integrity or on the efficiency of cell culture infection [1,2].

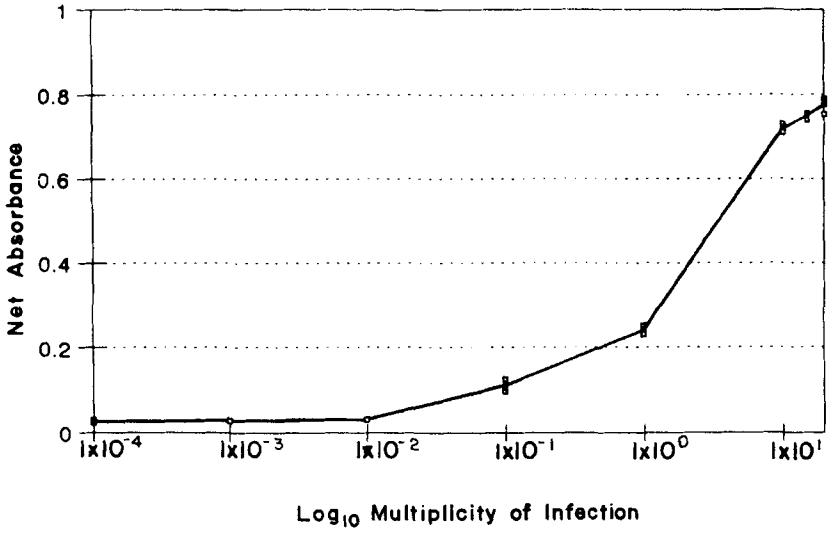

FIGURE 4. Quantification of cell-bound antigen in proteins extracted from infected cells. The plotted net absorbance values are the mean of three assays. The cut-off value was 0.042 OD taken as zero. Data points under $1X10^{-2}$ and over $1X10^{1}$ were omitted from the linear regression they were under the boundary of specific response. The correlation coefficient value was of 0.98.

However, at low antigen concentration it shows a non-linear response of the net absorbance values to antigen concentration, so that its use for antigen quantification requires a careful distinction between unspecific and specific readings. The reason for the non-linearity is not known.

We attempted to increase the sensitivity of the assay by using as label avidin/biotin instead of peroxidase. However, the background OD readings were to high to yield specific measurements. High levels of OD values were also observed initially with peroxidase. They were possibly due to non-specific binding of serum components to the viral antigen. However, by treating the sera with Vero cells the OD reading background was reduced to acceptable levels and the technique was specific as

evidenced by the correlation factor values obtained in some of the tests (Fig. 1, 2 and 4).

Although the sensitivity of the procedure in free antigen quantification ($1.5X10^2$ pfu/ml) was less than that obtained with the viral infectivity assay, we found the test very useful for routine work in the laboratory due to its capacity to quantify antigen at high concentrations. Results can be obtained in a few hours, total free antigen can be measured and neither cell cultures nor sterile working conditions are required.

The sensitivity of the technique depends on the antigen source. The highest values were obtained by quantification of cell bound antigen in attached cells. In these tests, at 24 and 48 h pi, sensitivity limits of $2X10^{-4}$ and $2X10^{-6}$ moi were obtained, respectively. Under these conditions, at 24 h, a minimum of 200 pfu/ml was required (infection of 10^6 cells). On the other hand, when the test was done after 48 h, viral antigen was detected at a multiplicity of $2X10^{-6}$, theoretically implying that two infective particles will be sufficient, though in practice more are required. Although it is difficult to quantify viral antigen by moi (neither the number of infected cells nor the amount of expressed viral antigen per cell are constant), the values of the correlation coefficient between net absorbance and antigen concentration evidenced the specificity of the procedure ($r=0.95$ and 0.8 for 24 and 48 h pi respectively).

Indirect ELISA could also be employed to quantify viral antigen in protein samples extracted from infected cells, though its sensitivity is low. Significant net absorbance values above the cut-off line were obtained first at moi of 10^{-3}, i.e. in infection of 10^6-10^7 cells a minimum of 10^3 to 10^4 infective particles is required. If the assay is performed after a longer period of infection, sensitivity might be higher.

Although the ELISA technique used to determine viral antigen in protein extract is laborious (every assay requires 10^6-10^7 infected cells), it has several advantages: it allows us to demonstrate the presence of viral antigen in the absence of a

manifest cytopathic effect and it could be used to determine HSV antigen in low concentrations where highly sensitive techniques such as polymerase chain reaction (PCR) are not available. Moreover, protein extract has its own useful application to protein analysis where PCR cannot yield information, e.g. simultaneous demonstration of several proteins on Western blots.

Another advantage of the described technique is that cell-viral antigen can be visualized, even when cytopathic effect is incipient.

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