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# DETECTION OF HERPESVIRUS ANTIGENS BY SOLID-PHASE RADIOIMMUNOASSAY WITH $^{125}$I-ANTIGLOBULIN AND $^{125}$I-PROTEIN A

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*Summary.* — The binding of immune (IS) and non-immune (NS) sera to herpes simplex virus type 1- (HSV-1) infected Vero cells was tested by indirect solid-phase radioimmunoassay (RIA) with radioiodinated swine anti-rabbit IgG ($^{125}$I-SwAR-IgG) or staphylococcal protein A ($^{125}$I-SPA). To indicate the binding of non-immune IgG molecules to virus-induced Fc-receptors, the cells were incubated in the presence or absence of the glycosylation inhibitor 2-deoxy-D-glucose (DOG). With $^{125}$I-SwAR-IgG, the binding of both IS and NS to untreated infected cells was higher at all time intervals than their binding to infected cells kept post infection in the presence of DOG. The titre of IS as detected by $^{125}$I-SwAR-IgG remained unchanged regardless whether the cells were incubated in the presence or absence of the drug. $^{125}$I-SPA gave much higher net binding than $^{125}$I-SwAR-IgG but, the end point titre of IS as measured by $^{125}$I-SPA was 1-2 dilution steps lower than with $^{125}$I-SwAR-IgG.

*Key words: herpes simplex virus; solid phase radioimmunoassay; radioiodinated staphylococcal protein A; radioiodinated anti-glubulin; 2-deoxy-D-glucose*

## *Introduction*

The highly sensitive indirect solid phase radioimmunoassay (RIA) technique has become widely used for measuring the extent to which antiviral antibody binds to virus-infected cells or immobilized viral antigens. The solid phase may involve surface areas of infected cells grown in test tubes (Forghani *et al.*, 1974, 1975) or in the wells of plastic microtiter plates (Jankowski *et al.*, 1977). Soluble viral antigens can be used for coating polystyrene beads (Kalimo *et al.*, 1977; Patterson *et al*, 1978), magnetic transfer devices (Smith and Gehle, 1977) or wells of microtiter plates (Rosenthal *et al.*, 1973; Colombat ti and Hilgers, 1979). The chief advantage of the solid-phase system is the

efficient washing and transfer of the antigen-bound antibody without centrifugation or filtration. The indirect RIA technique can be performed in two modifications. Most frequently the $^{125}$I-labelled IgG reacting with the immunoglobulin fraction of the antiserum in the first layer is utilized for quantitation of antigen-antibody reaction. Recently, staphylococcal protein A (SPA) which reacts with the Fc region of the IgG of most mammals (Lind *et al.*, 1970; Kronvall and Frommel, 1970) was introduced instead of the radiodinated antiglubulin. RIA with $^{125}$I-SPA has been recommended for measuring antibodies to oncoviruses (Colombatti and Hilgers, 1979), to evaluate antibody to serum phospholiphase (Hamilton *et al.*, 1979) and various other antigens (Marier *et al.*, 1979).

The solid-phase RIA with $^{125}$I-anti-IgG has been succesfully used for measuring antibodies to herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), for differentiation between the two serotypes and for selective measurements of IgG and IgM antibody responses (Forghani *et al.*, 1974, 1975; Kalimo *et al.*, 1977). Differences in the class-specific responses against crude and subunit HSV-1 antigens have also been described (Kalimo *et al.*, 1977).

The main problem of HSV serology with labelled antibodies is the false positive binding of the normal IgG to HSV-infected cells (Westmoreland and Watkins, 1974; Adler *et al.*, 1978). Because carbohydrate is involved in the synthesis of the receptor for IgG, we investigated the binding of normal serum (NS) and immune serum (IS) to HSV-infected cells incubated in the presence or absence of 2-deoxy-D-glucose (DOG). It is known that already at low concentration DOG interferes with glycosylation of precursor polypeptides (Courtney, 1976; McTaggart *et al.*, 1978).

We supposed that normal immunoglobulins bound to virus-induced Fc receptors (Costa *et al.*, 1978; Nakamura *et al.*, 1978; Rajčáni *et al.*, 1979) would not provide free binding sites for the attachment of $^{125}$I-SPA in contrary to $^{125}$I-SwAR-IgG. To elucidate this point, we compared the results of indirect RIA with $^{125}$I-SwAR-IgG and $^{125}$I-SPA as detecting reagents.

## *Materials and Methods*

*Virus.* The Kupka strain of HSV-1 was used throughout. The stock virus titered $5 \times 10^7$ plaque forming units (PFU) per ml.

*Cells.* Polystyrene microplates (Microtiter, Dynatech A. G.) were seeded with $3 \times 10^4$ Vero cells in 0.1 ml basal Eagle's medium (BEM) containig 10% inactivated calf serum (ICS). When the monolayers became confluent (within 40 hr), one half of the plates were incubated overnight in BEM + 2% ICS; the other half were kept in BEM/20 + 2% ICS (BEM/20 was prepared by mixing of 1 part of standard BEM with 19 parts of glucose-free BEM). Cells were infected with 0.1 ml HSV per well to achieve a multiplicity of 0.5 PFU/cell. After 60 min adsorption, the cells were washed with phosphate buffered saline (PBS). One half of the monolayers were then kept in BEM containing 2% ICS, while the other in BEM/20 + 2% ICS and 3.5 mM DOG. Mock-infected cells were treated in the same manner. All monolayers were incubated in a 3% $CO_2$ atmosphere at 37 °C. At intervals of 0, 4, 8, 24 and 32 hr post infection (p. i.), the wells were washed with PBS, fixed with 10% buffered formaldehyde for 20 min and stored at 4 °C.

*Sera* Hyperimmune serum (IS) to HSV-1 was prepared by immunization of rabbits with semi-purified virus prepared in a Ficoll gradient (Matis *et al.*, 1975). As measured by indirect im-

munofluorescence, the serum showed a staining titre of at least 1 : 640. Normal serum (NS) was obtained from a nonimmunized rabbit. The swine anti-rabbit immunoglobulin(SwAR-IgG) purchased from SEVAC (Prague) was purified on DEAE-cellulose (Levy and Sober, 1960) and its purity was then tested by immunoelectrophoresis.

*Labelling procedures.* SwAR-IgG and protein A (from *Staphylococcus aureus*; Pharmacia, Uppsala) were labelled with $^{125}I$ [Institute of Isotopes, Hungary; specific activity 110 mCi/ml

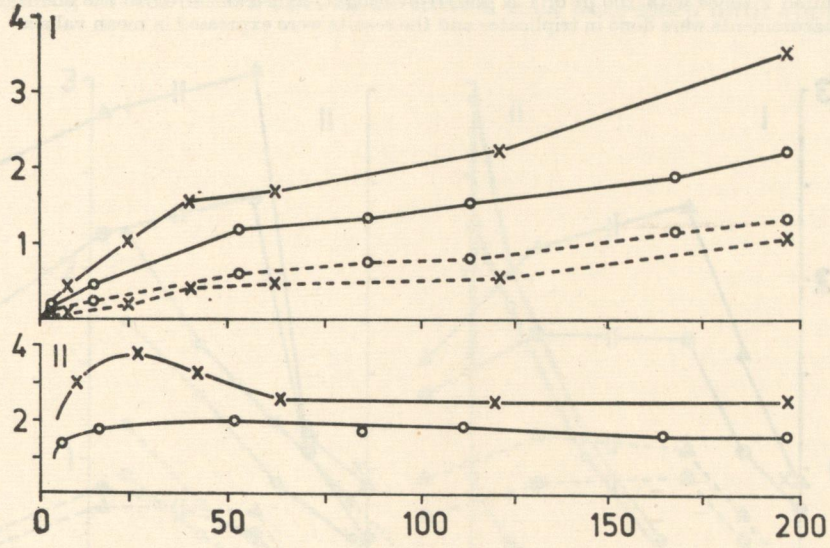


**Fig. 1.**

I —Plot of radiolabelled IgG and SPA bound to HSV-1-infected cells treated with IS and NS (diluted 1 : 100), against amounts of radiolabelled IgG and SPA added

× ——— × IS + $^{125}I$-SPA
× - - - - × NS + $^{125}I$-SPA
○ ——— ○ IS + $^{125}I$-SwAR-IgG
○ - - - - ○ NS + $^{125}I$-SwAR-IgG

Abscissa: count/min added × $10^{-3}$; ordinate: count/min bound × $10^{-3}$

II — Plot of the binding ratios of $^{125}I$-SPA (×) and $^{125}I$-SwAR-IgG (○) against the amounts of radiolabelled proteins

Abscissa: count/min added × 10−3; ordinate: binding ratio (count/min bound to IS-treated infected cells per count/min bound to NS-treated infected cells)

(4.07 GBq/ml)] by a modified chloramine-T method (Greenwood *et al.*, 1963). Briefly, equal volumes (50 µl) of SPA (0.5 mg/ml) and 0.5 M phosphate buffer, pH 7.5 were mixed in a glass vial. Then 10 µl chloramin-T (Serva) solution (5 mg/ml) and 5 µl Na $^{125}I$ (20.35 MBq) were added. After 90 seconds, 10 µl of a sodium metabisulfite solution (15 mg/ml) were added to stop the reaction. The mixture was applied to a Sephadex G-25 column saturated with bovine serum albumin (BSA) to remove the unreacted $^{125}I$. The reaction conditions for radiolabelling of SwAR-IgG were similar. To achieve the same molar protein to $^{125}I$ ratio as with SPA, IgG was used in a higher concentration (0.9 mg/ml), a greater volume of protein solution was added (100 µl), and the reaction time was prolonged (4 min). Under these conditions, the similar molar ratio of protein to $^{125}I$ was reflected by similar specific activities of the labelled proteins — 250 Ci/mmol (9.25 TBq/mmol). The radioiodinated proteins were diluted in 1% BSA (fraction V, Calbiochem) and stored at — 20 °C until used.

*RIA procedure.* Before addition of antibodies to the plastic wells, nonspecific sticking was prevented by incubation of the cell sheets with 200 μl PBS containing 5% BSA for 1 hr at 37 °C. Then 50 μl of diluted antiserum (1% BSA in PBS was used as diluent) were added and the plates were incubated for 1 hr at 37 °C. After washing (3 changes of PBS containing 0.1% BSA), 50 μl of $^{125}$I-SwAR-IgG or $^{125}$I-SPA were added and incubated for 1 hr at 37 °C. Unbound labelled proteins were removed by 3 washes with the washing buffer. Radioactivity bound to each well was eluted 2 times with 100 μl of 1 N NaOH overnight, and transferred to the counting vials. All measurements were done in triplicates and the results were expressed in mean values.

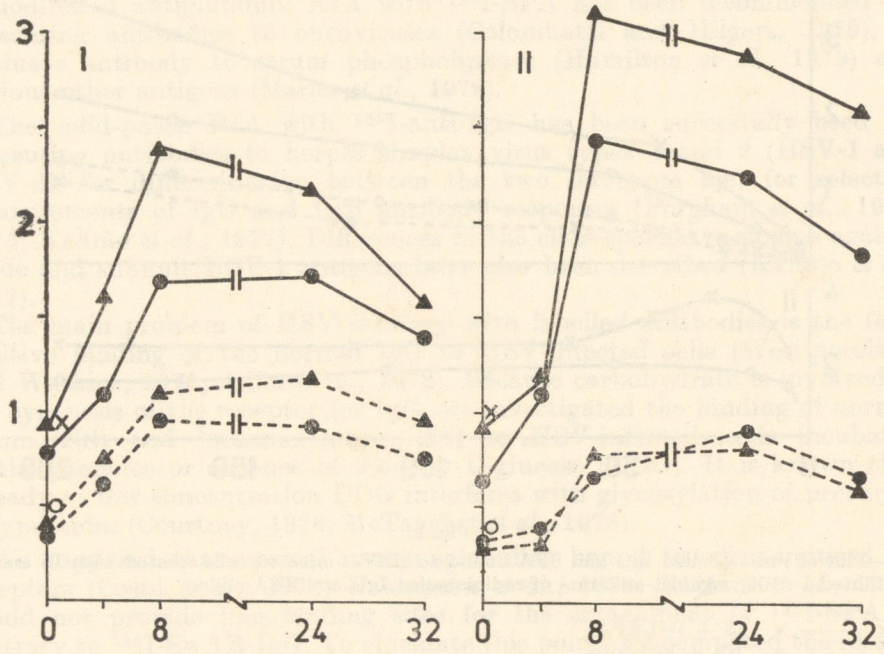


**Fig. 2.**

Binding of $^{125}$I-SwAR-IgG (I) and $^{125}$I-SPA (II) to HSV-1-infected cells at various intervals p. i. IS and NS in the first layer diluted 1 : 100 (I) and 1 : 50 (II)

▲——▲ IS, cells incubated in standard BEM
●——● IS, cells incubated in BEM/20 + DOG
▲- - - -▲ NS, cells incubated in standard BEM
●- - - -● NS, cells incubated in BEM/20 + DOG
× mock-infected cells treated with IS
○ mock-infected cells treated with NS
Abscissa: hours p. i.; ordinate: count/min × $10^{-3}$

## *Results*

### *Estimation of the optimal amount of radioiodinated SwAR-IgG and SPA added to NS and IS-treated cells*

The amount of radioactivity bound increased with increasing amounts of $^{125}$I-SwAR-IgG or $^{125}$I-SPA (diluted with 1% BSA) added to the HSV-

infected cells (incubated for 24 hr in BEM+2 % ICS), treated either with IS or NS (diluted 1 : 100) (Fig. 1-I). The binding of $^{125}$I-SPA to cells treated with IS was significantly higher in the entire concentration range tested, as compared to the binding of $^{125}$I-SwAR-IgG. On the other hand, the binding of $^{125}$I-SPA to NS-treated cells was slightly lower than that of $^{125}$I-SwAR-IgG. The binding curves of $^{125}$I-SPA to IS-treated cells were steep until approxi-

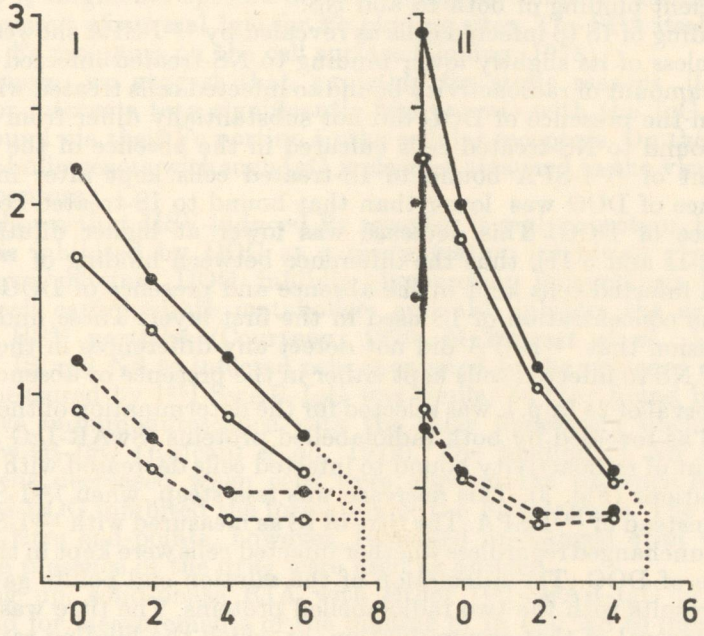


**Fig. 3.**

Binding of $^{125}$I-SwAR-IgG (I) and $^{125}$I-SPA (II) to HSV-1-infected cells (24 hr p.i.) at increasing serum dilutions

Abscissa: serum dilution ($\log_2 \times 10^2$); ordinate; count/min bound $\times 10^{-3}$

● ——— ● IS, cells incubated in standard BEM
○ ——— ○ IS, cells incubated in BEM/20 + DOG
● - - - - ● NS, cells incubated in standard BEM
○ - - - - ○ NS, cells incubated in BEM/20 + DOG

mately 40 $\times$ $10^3$ count/min were added. To achieve the same binding level, addition of 2-3 times more $^{125}$I-SwAR-IgG (112 $\times$ $10^3$ count/min) was needed. The highest binding ratio (ratio of radioactivity bound to IS-treated versus NS-treated infected cells) was reached for the $^{125}$I-SPA at 25 $\times 10^3$ count/min, while for $^{125}$I-SwAR-IgG at 50 $\times$ $10^3$ count/min (Fig. 1-II). Therefore, the amounts of radioactivity in the $^{125}$I-SPA and $^{125}$I-SwAR-IgG specimens were adjusted to contain this number of counts per 50 µl.

*Binding of IS and NS to HSV-1-infected cells at different intervals p. i. and at different serum dilutions as revealed by* $^{125}$*I-SwAR-IgG and* $^{125}$*I-SPA*

The binding of IS to infected cells as revealed by $^{125}$I-SwAR-IgG increased steeply between 4-8 hr p. i. and persisted at a similar level till 24 hr p. i. (Fig. 2-I). There was a slight binding of NS to infected cells, which increased from 4 to 8 hr p. i. and was clearly higher than the binding of NS to uninfected control cells. The infected cells incubated in the presence of DOG showed a less efficient binding of both IS and NS.

The binding of IS to infected cells as revealed by $^{125}$I-SPA showed a similar pattern unless of its slightly lower binding to NS-treated infected cells (Fig. 2-II). The amount of radioactivity bound to infected cells treated with NS and cultured in the presence of DOG did not substantially differ from the radioactivity bound to NS-treated cells cultured in the absence of the drug. But the amount of $^{125}$I-SPA bound to IS-treated cells kept after infection in the presence of DOG was lower than that bound to IS-treated cells kept in the absence of DOG. This decrease was lower at higher dilutions of IS (cf. Figs 2-II and 3-II), thus the difference between binding of $^{125}$I-SPA to IS-treated infected cells kept in the absence and presence of DOG depended also on the concentration of IS used in the first layer. These findings allow the conclusion that $^{125}$I-SPA did not detect any differences in the extent of binding of NS to infected cells kept either in the presence or absence of DOG.

The interval of 24 hr p. i. was selected for the determination of the end-point titre of IS as revealed by both radiolabelled proteins (SwAR-IgG and SPA). The amount of radioactivity bound to infected cells decreased with increasing serum dilutions (Fig. 3). This decrease was less steep, when $^{125}$I-SwAR-IgG was used instead of $^{125}$I-SPA. The titre of IS as measured with $^{125}$I-SwAR-IgG remained unchanged regardless whether infected cells were kept in the presence or absence of DOG. The calculation of the dilution end-points gave slightly different results with the two radiolabelled proteins. The titre was expressed as the reciprocal of that serum dilution, at which the binding ratio is equal to 1. Using this criterion, the titre of IS determined with $^{125}$I-SPA was at least 1-2 dilution steps lower (3200) than with $^{125}$I-SwAR-IgG (12800).

## *Discussion*

In the present experiments the results of indirect non-competitive solid-phase RIA procedure with radioiodinated SwAR-IgG and SPA were compared. The common advantage of SPA is that it can be used for measuring the binding of the majority of mammalian immunoglobulins in contrary to the species-specific antiglobulin. It has been shown that SPA binds specifically to the Fc region of immunoglobulins (Kronvall and Frommel, 1970). Because of the high avidity of this reaction, the net binding of $^{125}$I-SPA was much higher than that of $^{125}$I-SwAR-IgG, but it showed a strong dependence on the serum dilution used in the first layer. As to sensitivity, it was evident from serum titrations that radioiodinated SPA was less sensitive than radioiodinated SwAR-IgG. This is consistent with the notion that there is

one binding site at the interface between the $C_H2$ and $C_H3$ domains of the rabbit IgG molecule, which can react with SPA, whereas anti immunoglobulin usually reacts with several determinants on light and heavy chains of IgG (Lancet *et al.*, 1978).

Cells infected with herpesviruses develop an affinity for normal IgG of several species, which is directed specifically toward the Fc portion of the molecule (Westmoreland and Watkins, 1974). Adler et al. (1978) found that the F $(ab')_2$ fragments specific of HSV antigens competed efficiently with the Fc fragment of normal IgG for Fc binding sites. The SPA itself does not react with Fc receptors on the cell surface (Goding, 1978).

Furthermore, we suggest that, probably for steric reasons, it does not interact (or interacts to a significantly less extent) with the IgG molecules already bound via their Fc portion to the cellular receptors. On the contrary, the antiglobulin reacts with such IgG molecules attached to the virus-induced surface receptors.

It was shown that HSV-induced Fc receptor is a glycoprotein, because its synthesis is inhibited by DOG at a concentration inhibiting glycoproteins but not total poteins. DOG inhibits glycoprotein biosynthesis interfering with the cell carbohydrate metabolism and also inhibits the synthesis of infectious HSV particles (Courtney, 1976; McTaggart *et al.*, 1978). The binding of IS and NS to infected cells incubated in the presence or absence DOG as measured by $^{125}$I-SwAR-IgG was similar to that found by indirect quantitative immunoflurescence with the same fluorescein isothiocyanate-labelled SwAR-IgG (Rajčáni *et al.*, 1979). The binding of NS and IS to infected cells decreased when cells were kept in the presence of 3.5 mM DOG. Thus DOG inhibited the formation of the Fc receptors. The reading of the serum titre end-points, however, remained unchanged even when cells kept in the presence of the drug were used as antigen.

Summing up, solid-phase RIA with either $^{125}$I-SwAR-IgG or $^{125}$I-SPA can be used for measurements of the binding of IS and NS to infected cells. The advantage of $^{125}$I-SPA is a more accurate detection of the immune IgG molecules bound to herpes-virus-infected cells, because these cells regularly develop binding sites for the Fc portion of the nonimmune IgG.

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