Enhancement by Phytohaemagglutinin of Inactivation of Herpes Simplex Virus by Concanavalin A

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SUMMARY

Inactivation of herpes simplex virus (HSV) by concanavalin A (Con A) was enhanced by treatment with phytohaemagglutinin (PHA)-P using a two-stage reaction procedure. Treatment with PHA alone failed to inactivate HSV. Enhancement of inactivation was also effective when HSV was exposed to PHA first. Our results suggest that the envelope of HSV contains receptor sites for Con A which are important in the infectious process, as well as receptor sites for PHA which are not critical for infectivity. Direct interaction of Con A with PHA was demonstrated and the reaction was reversed by α-methyl-d-glucoside. The data indicate that PHA stabilized the binding of Con A to the virus since reversal of inactivation by α-methyl-d-glucoside was minimal following treatment with Con A and PHA. Con A inactivated only enveloped virions and enhancement by PHA was a general phenomenon.

INTRODUCTION

Lectins which are capable of combining with specific carbohydrate determinants on the surface of animal cells have been isolated from a variety of plants and lower animals (Lis & Sharon, 1973; Nicolson, 1974). It has been reported that certain lectins such as concanavalin A (Con A) can affect virus replication especially by blocking release of enveloped viruses (Rott et al. 1972) as well as the appearance of c.p.e. (Reeve et al. 1972; Poste et al. 1974). Binding of Con A to the cells prevented the entry of certain viruses into the cells (Okada & Kim, 1972). Ishizaki & Bolognesi (1976) have reported that avian RNA tumour viruses are inactivated by Con A or phytohaemagglutinin (PHA) but not by wheat germ agglutinin. In our previous paper (Ito & Barron, 1974) we reported that Con A inactivated herpes simplex virus (HSV) whereas PHA failed to affect infectivity.

In this communication we describe the results of experiments demonstrating enhanced inactivation of HSV by Con A with PHA. Evidence obtained for the presence of PHA-binding sites on the envelope of virions is reported as well.

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METHODS

Cells. A continuous line from rabbit kidney (BIRK) was used to prepare virus stocks (Ito & Barron, 1972a). Cells were grown in Eagle's basal medium in Hanks' balanced salt solution containing 10% foetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). A cell line from African green monkey kidney (BGM) was employed for plaque assays (Barron, Olshevsky & Cohen, 1970). BGM cells were cultivated in Eagle's minimal essential medium (MEM) in Earle's balanced salt solution (bicarbonate, 18 mg/ml) supplemented with 10% foetal calf serum and antibiotics. After virus inoculation, cells of both lines were maintained in MEM containing 2 to 3% serum and antibiotics.

Viruses. Herpes simplex type 1 (HSV-1), MacIntyre VR 3 strain; pseudorabies virus, Aujesky strain; vesicular stomatitis virus (VSV), Indiana strain; and vaccinia virus, NY strain have been described previously (Ito & Barron, 1972a, b, 1974). HSV-2, strain 3016, was a fresh isolate from genital infection obtained from the Erie County Virology Laboratory, Buffalo, New York. The virus was passaged three times in BIRK cells and used for experiments.

Infectivity assay. Plaque titration was performed in BGM cells grown in 30 ml plastic flasks as reported previously (Ito & Barron, 1972a, 1974). Phosphate buffered saline containing 0.3 mM-CaCl₂ and 0.05 mM-MgCl₂ (PBS) was employed as diluent. Daniel's modified Eagle's medium (Grand Island Biological Company, Grand Island, New York) containing 3% foetal calf serum and 0.7% agarose (Seakem, Bausch and Lomb, Rochester, New York) was used as an overlay medium for plaque assay of herpesviruses. The agarose was replaced by 1.2% Noble agar (Difco, Detroit, Michigan) for assay of the other viruses. After incubation at 36 °C for appropriate intervals, BGM cells were stained with a second overlay medium containing 0.007% neutral red.

Chemicals. Con A was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio (lot no. 5594, crystallized twice). Phytohaemagglutinin-P, PHA-P (Difco), Phytohaemagglutinin-M, PHA-M (GIBCO), Pokeweed mitogen (GIBCO) were prepared according to the suppliers and were considered as undiluted in our experiments. Crude wheat germ agglutinin was prepared from wheat germ lipase (Schwartz Mann, Orangeburg, New York; Aub, Sanford & Cote, 1965). Leucoagglutinin, one component of PHA-P, was purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. All the lectins employed except wheat germ agglutinin were used without further purification in our laboratory. α-methyl-d-glucoside was obtained from Sigma Chemical Company, St Louis, Missouri.

Treatment of viruses with lectins (two-stage procedure). Equal volumes of appropriate concentrations of Con A or other lectins and mitogens in PBS were mixed with viruses and incubated 1 h at 25 °C (room temperature) in stage 1. Thereafter, the mixtures were diluted at least 100 times in PBS and equal volumes of either lectins or PBS were added in stage 2. These mixtures were further incubated for 1 h at 25 °C and residual infectivity was assayed by plaque method.

Since variation in infectivity titres was observed from one experiment to another possibly due to Con A inhibitors present in the assay system calculation of valid means and standard errors was not possible. Each experiment was performed at least twice and the pattern of results was very reproducible.

Double diffusion in gel. Interaction of lectins with each other was examined by the method of double diffusion in gel established in our laboratory (Barron, 1971). Microscope slides were coated with 0.7% agarose dissolved in 0.15 M-saline. Wells (3.0 mm in diam.) were
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punched in the agarose layer and charged with reagents. The slides were incubated at 25 °C and examined periodically for the appearance of reaction lines.

Con A-Sepharose column chromatography. Con A-Sepharose was prepared following the method described by Kristiansen, Sundberg & Porath (1969) with slight modifications. CNBr (Schwartz Mann, Orangeburg, New York) was dissolved in distilled water and mixed with Sepharose 4B (Sigma) plus distilled water with continuous stirring at 25 °C. The mixture was equilibrated for 1 h with stirring before activation by continuous addition of 5 N-NaOH to maintain pH at 11·0 to 11·2 for 8 min. The activated Sepharose was quickly washed with ice water followed by ice-cold 0·1 M-NaHCO₃, pH 8·5, on a glass filter funnel and immediately suspended in 0·1 M-NaHCO₃ solution, to which was added Con A in NaHCO₃ solution. The mixture was stirred overnight at 4 °C. The Con A-coupled Sepharose was washed again with 0·1 M-NaHCO₃ and water on a glass filter and packed in a glass column (9 × 150 mm). The Con A-Sepharose column was washed with 0·1 M-NaHCO₃, 1 M-NaCl, distilled water, PBS, 0·3 M-α-methyl-D-glucoside in PBS and finally with PBS. The material in PBS was applied on the top of the column and allowed to adsorb to the Con A-Sepharose particles. Substances which did not react with Con A were passed through the column by continuous flow of PBS. Eluates were obtained by the addition of 0·3 M-α-methyl-D-glucoside in PBS and fractions (2·0 ml each) were collected.

PHA was detected by haemagglutination (HA) of sheep red blood cells (SRBC) measured by the microtitre method using a disposable plastic tray. One drop (0·025 ml) of serial twofold dilutions of materials in PBS was mixed with an equal volume of 0·8 % SRBC. HA patterns were read following incubation for 1 h at 25 °C.

Treatment of BGM cells with lectins. BGM cell monolayers in flasks were exposed to Con A (0·1 mg/flask) or PHA-P (0·2 ml/flask of 1:16 dilution) for 30 min at 25 °C. The cell sheets were washed 3 times with PBS and inoculated with HSV-1 previously treated with either PBS, Con A (0·1 mg/ml), or PHA-P (1:16) for 1 h at 25 °C. Following incubation at 25 °C for 2 h, the cells were overlayed for plaque assay.

Reversal of inactivation of HSV-1. Appropriate dilutions of HSV-1 were treated with either Con A (0·3 mg/ml): PBS, Con A: PHA-P (1:256), or PBS: PBS by the two-stage procedure. Thereafter, the virus mixtures were exposed to α-methyl-D-glucoside, final concentration 0·1 M, for 30 min at 25 °C, and directly assayed for infectivity with PBS as diluent.

Recovery of HSV-1 inactivated by Con A from cell cultures. HSV-1 was treated with lectins or PBS as described above in reversal section; BGM cells were inoculated and incubated for 2 h at 25 °C to permit adsorption of virus. Inoculum was discarded and the cells were washed three times with PBS. α-methyl-D-glucoside (0·5 ml per flask of 0·1 M-solution) was added to appropriate flasks which were further incubated for 30 min at 25 °C. Flasks were then overlaid to assay for infectivity.

RESULTS

Enhancement of inactivation of HSV-1 by Con A with PHA using two-stage procedure

As reported previously (Ito & Barron, 1974) HSV-1 was inactivated following treatment with Con A, but not with PHA, wheat-germ agglutinin or pokeweed mitogen. As shown in Table 1, approx. 1 % of infectivity was always recovered following exposure to Con A, and further treatment with Con A failed to reduce this residual infectivity. When PHA was added at stage 2, the inactivation of HSV-1 was markedly enhanced and residual infectivity...
Table 1. The effect of plant lectins and mitogens on the infectivity of HSV-1 previously treated with Con A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infectivity of HSV-1</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>p.f.u.</td>
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<tr>
<td>Stage 1</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>14000</td>
</tr>
<tr>
<td>Con A (0.5 mg/ml)</td>
<td>2100</td>
</tr>
<tr>
<td>PHA-P (1:256)</td>
<td>27</td>
</tr>
<tr>
<td>Wheat-germ agglutinin (10 mg/ml)</td>
<td>4800</td>
</tr>
<tr>
<td>Pokeweed mitogen (1:1)</td>
<td>4080</td>
</tr>
</tbody>
</table>

* Two experiments were performed with very similar results. One was selected for presentation in the table.
† Treatment of HSV-1 by lectins by two-stage procedure. See Methods section.

was decreased from 1.3 to 0.02%. Among the lectins and mitogens tested, only PHA-P was found to increase inactivation by Con A since wheat-germ agglutinin and pokeweed mitogen had no enhancing effect. Therefore, the enhancement by PHA could be considered specific. Other combinations of lectins such as PHA: wheat germ agglutinin, PHA: pokeweed mitogen and wheat-germ agglutinin:pokeweed mitogen were also tested, and no significant loss of infectivity of HSV-1 was obtained.

Varying concentrations of PHA-P were examined for enhancement of inactivation using the two-stage procedure. In one experiment, dilution of the preparation to 1:4096 resulted in a decrease in residual infectivity from 3.3 to 0.07%. PHA-M, a less purified form than PHA-P, and leucoagglutinin, containing one purified component from PHA-P, were also effective in enhancing inactivation by Con-A.

If the two-stage procedure was reversed and the virus first treated with PHA, enhancement of inactivation was also observed. In one experiment residual infectivity was reduced from 3.2 to 0.015% by pre-treatment with PHA-P 1:1. This procedure may have been less efficient in that under these conditions dilution of PHA-P 1:4096 did not enhance inactivation by Con A. However, this interpretation is difficult to establish in view of the differences in the conditions.

Direct interaction between Con A and PHA

Simultaneous treatment of HSV-1 with Con A and PHA failed to enhance inactivation by Con A. These results lead to experiments to study the direct interaction of the two lectins.

Interaction of PHA with Con A was tested using double diffusion in agarose gel. A distinct precipitation line was observed between PHA-P (1:10) and Con A (1 mg/ml), but no reactions were detectable either between PHA-P and wheat-germ agglutinin (10 mg/ml) or PHA-P and pokeweed mitogen (1:1).

The direct interaction of PHA-P and Con A was also studied by use of affinity chromatography with a Con A-Sepharose column (Fig. 1). PHA was completely adsorbed on the Con A-coupled Sepharose and no PHA, as detected by HA activity, was found in PBS fractions. Almost 100% of PHA could be recovered in a sharp single peak after replacing PBS with 0.3 M-α-methyl-D-glucoside (see below) in PBS. These results clearly indicated that PHA-P could bind to Con A molecules directly. PHA eluates from a Con A-Sepharose column obtained following treatment with α-methyl-D-glucoside enhanced Con A inactivation of HSV-1 (data not shown). No enhancement was obtained with the PBS fractions.

In the two-stage procedure the critical step was considered to be dilution of the Con A-
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Fig. 1. Affinity chromatography of PHA-P on a column containing Con A-coupled Sepharose. PHA-P (0·5 ml of 1:4 dilution) was passed at 25 °C over a column (9 x 150 mm), which had been equilibrated with PBS (pH 7·2). The column was washed with PBS (A) and elution was achieved with 0·3 M-α-methyl-D-glucoside in PBS (B). Fractions (2·0 ml) were collected for measuring HA titre.

Table 2. Effect of α-methyl-D-glucoside on recovering infectivity of HSV-1 directly from virus-lectin mixtures or from BGM cell cultures inoculated with virus-lectin mixtures*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>α-methyl-D-glucoside</th>
<th>Recovery HSV-1 infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>PBS</td>
<td>−</td>
<td>BGM cultures inoculated</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>PBS</td>
<td>+</td>
<td>with virus-lectin</td>
</tr>
<tr>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>−</td>
<td>1 15000†</td>
</tr>
<tr>
<td>Con A (0·3 mg/ml)</td>
<td>PBS</td>
<td>PBS</td>
<td>+</td>
<td>50 000</td>
</tr>
<tr>
<td>Con A (0·3 mg/ml)</td>
<td>PBS</td>
<td>PHA-P (1:256)</td>
<td>−</td>
<td>92 000</td>
</tr>
<tr>
<td>Con A (0·3 mg/ml)</td>
<td>PBS</td>
<td>PHA-P (1:256)</td>
<td>+</td>
<td>27 800</td>
</tr>
</tbody>
</table>

* Three experiments were performed with similar results and one was selected for presentation.
† p.f.u.

virus mixture to dilute the Con A sufficiently to avoid conditions for formation of precipitates upon addition of the PHA-P.

Pre-treatment of cells with either Con A or PHA

As previously reported (Okada & Kim, 1972; Ito & Barron, 1974) pre-treatment of cells resulted in decreasing infectivity of HSV-1. In the present study enhancement of inactivation of HSV-1 occurred if BGM cells were pre-treated with either Con A or PHA and the virus treated with the alternate lectin. As an example, in one experiment pre-treatment of cells with Con A and treatment of virus with PHA-P 1:16 resulted in residual infectivity of 0·04 % whereas if Con A alone was used to treat cells and virus, residual infectivity was 1·7 %.
Table 3. Enhancement of inactivation of enveloped viruses by Con A treatment with PHA*

<table>
<thead>
<tr>
<th>Two-stage treatment</th>
<th>Enveloped</th>
<th>Non-enveloped</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Herpesvirus</td>
<td>Rhabdovirus</td>
</tr>
<tr>
<td>PBS: PBS</td>
<td>HSV-1</td>
<td>HSV-2</td>
</tr>
<tr>
<td>Con A: PBS§</td>
<td>100‡</td>
<td>100</td>
</tr>
<tr>
<td>Con A: PHA-P§</td>
<td>0·02</td>
<td>0·006</td>
</tr>
</tbody>
</table>

* Two experiments were performed with similar results and one was selected for presentation.
† Pseudorabies.
‡ Infectivity (%).§ Con A, 50 mg/ml; PHA-P, 1:16.

Effect of α-methyl-D-glucoside
Inactivation of HSV-1 by Con A has been shown to be a reversible phenomenon (Okada & Kim, 1972; Ponce de Leon, Hessle & Cohen, 1973; Ito & Barron, 1974). If specific sugars such as α-methyl-D-glucoside were added to HSV-Con A mixtures, inactivation was easily reversed (Table 2). However, in the case of HSV-1 previously treated with Con A and PHA in the two-stage procedure, reversal of inactivation was not obtained. Our previous studies (Ito & Barron, 1974) also demonstrated that Con A-inactivated virus could adsorb to BGM cells and additions of α-methyl-D-glucoside to cell monolayers resulted in recovery of infectious virus (Table 2). Recovery of infectivity from BGM cells inoculated with HSV-Con A: PHA following treatment with α-methyl-D-glucoside was minimal. These results indicated that inactivation of HSV-1 by Con A was rendered irreversible following treatment with PHA. It is possible that HSV-Con A: PHA did not adsorb to the cells but no data are available on this point.

Enhancement of inactivation of enveloped viruses by Con A by treatment with PHA
It has been reported that Con A will inactivate a number of enveloped viruses belonging to different groups (Okada & Kim, 1972; Ito & Barron, 1974). It was considered of interest to learn if the enhancement of inactivation of HSV-1 observed in this study had broad application among enveloped viruses (Table 3). As can be seen, enhancement of inactivation was observed for other herpesviruses as well as for VSV, a member of the rhabdovirus group. Inactivation did not result upon treatment of vaccinia virus with Con A nor was the situation altered by addition of PHA.

DISCUSSION
Our previous studies (Ito & Barron, 1974) revealed that the aggregation of virus particles was not a major mechanism for inactivation of HSV-1 by Con A. The data obtained for enhancement of this inactivation by PHA in the present report do not directly exclude aggregation. It is noteworthy that interaction of HSV-1, Con A and PHA resulted in a stable inactivated complex while Con A directly reacted with PHA alone resulted in complexes that could be reversed by simple sugars. Thus, the phenomenon of enhancement may be more involved than just simple aggregation. Further experimentation is needed to provide a complete explanation.

A phenomenon similar to virus neutralization was suggested to occur in HSV-1 and Con A mixtures. It has been demonstrated that the neutralization of virus infectivity by
specific antibodies could be potentiated by the addition of anti-immunoglobulin G (IgG) (Ashe & Notkins, 1966) or complement (Yoshino & Taniguchi, 1965). Such enhancement of neutralization only occurred either when HSV was first reacted with corresponding antibody followed by anti-IgG or when the virus was simultaneously treated with virus antibody and complement. Enhancement was not demonstrated when the virus was first exposed to anti-IgG or complement, diluted and reacted with antiviral antibody. An explanation for this would be that virus envelopes do not contain reactive sites for anti-IgG or complement.

Enhancement of inactivation by Con A with PHA, however, occurred at almost the same efficiency regardless of the order of HSV-1 exposure to either lectin. These data suggest the presence of PHA-binding sites on the envelope of HSV virions along with Con A receptor sites. PHA-binding sites do not appear to be critical for the infectivity of HSV, since PHA alone failed to inactivate the virus. At present little is known about properties of PHA-binding sites located on the envelope, but it could be speculated that they are glycoproteins or glycolipids similar in nature to Con A receptors. Our previous data (Ito & Barron, 1974) also proved that Con A-inactivated HSV was capable of adsorbing to host cells and blockade of infectivity appeared to occur at the step of penetration or uncoating. This is similar to the behaviour of viruses which have been neutralized by their corresponding antibodies in that they can adsorb to cells but not penetrate or uncoat (Sehag, 1968). Thus, studies of interaction between lectins and viruses may provide a novel approach for investigation of virus neutralization by antibodies.

A Con A-resistant fraction which consisted of 0.2 to 4% of the virus population was constantly detected following treatment with Con A (Ito & Barron, 1974). As shown here, the resistant fraction could be reduced markedly by further exposure to PHA. We originally speculated that the majority of virions which compose the Con A resistant fraction possess receptor sites for Con A and that interaction does occur. These virions adsorb to the cells but infectivity is reactivated perhaps by Con A inhibitors present on the cell surface. Data obtained in the present study with PHA appear to support the above hypothesis. Very small portions, approx. 0.01% or less, of the original infectivity of HSV-1 was still recovered following exposure to Con A and PHA. This residual infectivity may be associated with virus particles which do not have Con A receptors, although direct evidence to support this is lacking at present.

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REFERENCES


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