Concanavalin A-Mediated Agglutination of 3T3 Cells After Exposure to UV-Irradiated Herpes Simplex Virus

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Abstract Studies were made to determine the effect of UV-irradiation of herpes simplex virus (HSV) on Concanavalin A (Con-A)-mediated agglutination of 3T3 cells. There were three different phases of agglutination by Con-A of cells infected with HSV. The agglutinability began to increase from 3 or 4 hr, or 72 hr after exposure of cells to HSV. The early-appearing agglutinability was further divided into two phases, based on its sensitivity to metabolic inhibitors. These were tentatively called “Early 1 or inhibitor sensitive”, “Early 2 or inhibitor insensitive” and “Late” agglutinability.

“Early 1” agglutination, detected from 3 hr post infection (pi), was induced by treating cells with HSV, either active or UV-irradiated for less than 5 min and was inhibited when actinomycin D (1 µg/ml) or cycloheximide (50 µg/ml) was added to the cultures. “Early 2” agglutination began to increase from 4 hr pi when cells were inoculated with HSV irradiated for 7 to 20 min and was not affected by either inhibitor. HSV irradiated for 6 min failed to induce either agglutinability. “Late” agglutination, observed 72 hr pi, was detected in cultures which had been treated with HSV irradiated for 4 to 15 min. Among those, virus irradiated for 6 to 8 min was most efficient. HSV-transformed cells were also agglutinated without exception by low concentrations of Con-A.

When mammalian cells in culture are infected with oncogenic DNA viruses, these cells can be agglutinated upon treatment with plant lectins under conditions of active host-DNA synthesis and cell replication (1, 18). Cells which were infected with or transformed by herpes simplex virus (HSV) could be agglutinated by low concentration of Concanavalin A (Con-A) (20, 21). Furthermore, it was found that replication of HSV in the cells was not required in order to produce Con-A-mediated agglutinability, because cells exposed to UV-inactivated HSV were regularly agglutinated by Con-A (14, 20).

It was demonstrated by several investigators that biochemical transformation (4, 13) or morphological transformation (2, 3, 5–7) could be induced by exposure of cells to UV-inactivated HSV. The frequency of this transformation was influenced, however, by the length of time when the virus was exposed to UV-irradiation (2, 8). Present experiments were made to determine the effects of UV-irradiation of HSV on Con-A-mediated agglutination of 3T3 cells.
MATERIALS AND METHODS

**Virus.** HSV type 2, strain 196, was used. Stock virus was harvested from Vero cell cultures. Supernatant from uninfected Vero cultures was served as control. Preparations were stored at -70°C until used. Infectivity of virus stocks was about $5 \times 10^7$ PFU (plaque forming units) per ml.

**Cell cultures.** Vero and Swiss/3T3 cell lines were used. Vero cell cultures were utilized for preparing stock HSV and for plaque assay. Swiss/3T3 cells were used in agglutination experiments. Both cell lines were propagated in a medium consisting of Eagle's minimum essential medium (MEM) containing 60 μg/ml Kanamycin, 0.1% sodium bicarbonate and 10% heat-inactivated calf serum. Care was taken to maintain monolayers of 3T3 cells at a constant density of $1.1$ to $1.2 \times 10^6$ cells per plastic plate (60-mm diameter).

**Infection of cells with HSV.** 3T3 cells were seeded into plastic plates. After growth at 37°C for 3 days, monolayers were semi-confluent, with a suitable cell density (1.0 to $1.1 \times 10^6$ cells per plate). Cells were also seeded into 4-oz prescription bottles. Unless otherwise stated, HSV was inoculated into cultures at a multiplicity of infection (moi) of 2 PFU or its equivalent per cell, and virus was adsorbed to cells for 90 min at 4°C or 37°C. Cells were washed once with phosphate buffered saline (PBS), then refed with MEM containing 2% calf serum. Control cells were similarly treated with Vero culture fluid being substituted for the virus inoculum.

**Chemicals.** Crystalized 3 times and then lyophilized Con-A (Miles-Yada Co. Israel), cycloheximide (Sigma Chemical Co., U.S.A.), and actinomycin D (Makor Chemicals, Israel) were used.

**UV-irradiation.** UV-irradiation was done in a dark room using a 10-watt germicidal lamp (Hitachi Co.) at a distance of 15 cm. Irradiation dosage was approximately 45 ergs/mm²/sec. The inactivation rate of HSV was determined by counting plaques of surviving virus in Vero cells. One- or 2-min irradiation, for example, resulted in inactivation of 99.9% or 99.99% of the virus initially presented. When Vero cells were inoculated with 1.0 ml of undiluted HSV which had been exposed to UV for 4 min (UV4-HSV), only 4 plaques were counted. With UV5-HSV, no plaque appeared. In 3T3 cells, on the other hand, live HSV induced cytopathic effect (CPE) between 8 and 10 hr pi, but no progeny was produced. Failure of HSV to replicate in 3T3 cells was previously reported by Duff and Rapp (8).

**Cell agglutination test.** As reported previously (20), 3T3 cells infected with HSV at a moi of 2 were dispersed with 0.02% EDTA in PBS, and resuspended in PBS at a concentration of about $1 \times 10^6$ cells/ml. The final concentration of stock Con-A was determined photometrically (19). For assay, 0.15 ml of cell suspension was mixed with an equal volume of serial 2-fold dilutions of Con-A in plastic plates with flat-bottomed wells (Linbro Scientific Co., U.S.A.). Plates were gently shaken for 30 min at room temperature, and the degree of cell aggregation was observed under a microscope. Grading was from negative (agglutination of less than 20% of the cells)
to 4+ (agglutination of more than 80% of the cells with the appearance of large cell clumps). The minimal Con-A concentration at which cells showed a 4+ agglutination was considered as a positive end-point and results are reported in terms of agglutinability by given concentrations of Con-A. Competing controls consisted of cells with or without HSV plus a mixture of 1,000 μg/ml of Con-A and 0.1 mM α-methyl-d-glucopyranoside (MGP), and of cells plus PBS only.

Cell transformation assay. The methods for quantitative assay of morphologically transformed cells were described by Duff and Rapp (8). One million 3T3 cells were infected in suspension with HSV at a moi of 5 PFU or its equivalent per cells, seeded in ten 60-mm plastic plates at 5 × 10⁴ cells per plate and fed with 5 ml of MEM containing 10% calf serum. Plates were incubated in a CO₂ incubator at 37 C for 3 to 4 weeks. The number of transformed foci was determined microscopically. The frequency of cell transformation was expressed as a ratio of PFU/FFU, where PFU indicates plaque forming units per 1 ml of the stock virus before UV-irradiation, and FFU indicates focus forming units. FFU values are calculated from the average number of the foci per plate, based on results from 3 to 4 repeated experiments, multiplied by a virus dilution factor for HSV inoculum in order to express the results as FFU per 1 ml of the virus before dilution.

RESULTS

Kinetics of Con-A-Mediated Agglutinability of HSV-Inoculated Cells

Groups of 4-oz bottle cultures of 3T3 cells were infected with HSV, either active (UV0), UV-irradiated for 6 min (UV6) or for 20 min (UV20). Virus was adsorbed to cells for 90 min at 4 C in order to synchronize the penetration. After adsorption (time 0) all cultures were washed with cold MEM. Two bottles of each

![Fig. 1. Kinetics of agglutination with Con-A of HSV-inoculated 3T3 cells. Duplicate cultures were inoculated at a moi of 2 with HSV which had been irradiated for 0 min (UV0-HSV), 6 min (UV6-HSV) or 20 min (UV20-HSV). Cells were incubated for 90 min at 4 C (time 0), fed with prewarmed medium, incubated at 37 C for the time indicated, and observed for agglutination after addition of Con-A in various concentrations (maximal 4+).](image-url)
group were examined for cell agglutination by Con-A at time 0. The remaining cultures were incubated at 37°C after prewarmed medium was added.

Figure 1 illustrates the kinetics of cell agglutinability. Cells exposed to UV20-HSV, UV6-HSV and UV0-HSV were maximally agglutinated by Con-A at time 0 at concentrations of 31 μg/ml, 62 μg/ml, and 125 μg/ml, respectively. The time course of changes of agglutinability was characterized by 1) a marked decrease in the magnitude of cell agglutinability during the first 2 hr incubation at 37°C of the cells exposed to HSV, either active or irradiated, and then, 2) a decrease in agglutinability of UV6-HSV cells after 4 hr or longer period incubation to the level of the control (1,000 μg/ml of Con-A), 3) a marked increase in UV0-HSV cell agglutinability after 3 hr or thereafter to the level of 62 μg/ml of Con-A, and 4) a slower increase in agglutinability of UV20-HSV cells, than that of UV0-HSV after 4 hr or thereafter up to 31 μg/ml of Con-A. Mock-infected cells were consistently agglutinated only by 1,000 μg/ml of Con-A, and competing controls were always run along with a mixture of Con-A and MGP and resulted in negative agglutination. The results at time 0 would indicate that the virus itself, or the cell surface modified by the virus, contributes in part to the agglutination phenomenon. Furthermore, appearance of agglutinability of the cells was significantly affected by the duration of UV-irradiation of the virus inoculum.

**Effect of UV-Irradiation Time of HSV on Cell Agglutination**

3T3 cells were inoculated with HSV which had been irradiated for various periods of time, and agglutinability of the cells was tested 6 hr pi. As shown in Fig. 2, UV-irradiation of HSV from 1 to 6 min resulted in a stepwise decrease in agglutinability in terms of the concentration of Con-A required to produce a 4+ agglutination. When virus was irradiated longer, there was a marked increase in
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Cell agglutinability (from 500 to 31 μg/ml of Con-A) to reach maximal agglutination (31 μg/ml Con-A) by the virus irradiated for 10 min or longer. These results suggest that Con-A-mediated agglutination might not be caused by action of only one of HSV-functions. Thus, it is of interest to determine whether macromolecule synthesis in the cells are required to bring about surface changes leading to agglutinability with Con-A, when cells were infected with HSV which had been irradiated by UV for a rather longer duration such as for 20 min.

Effect of Metabolic Inhibitors on Cell Agglutination

Before or just after infection, cells were treated with 1 μg/ml of actinomycin D or 50 μg/ml of cycloheximide to determine the effect of alteration in macromolecule synthesis on cell agglutination. Results shown in Table 1 indicate that cells infected

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Agglutination of cells infected with</th>
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<tbody>
<tr>
<td></td>
<td>UV0-HSV</td>
</tr>
<tr>
<td>Medium alone</td>
<td>4+</td>
</tr>
<tr>
<td>Actinomycin D, 1 μg/ml</td>
<td>2 hr to +5 hr</td>
</tr>
<tr>
<td>Cycloheximide, 50 μg/ml</td>
<td>2 hr to +5 hr</td>
</tr>
</tbody>
</table>

3T3 cells were inoculated with either HSV (active [UV0] or UV-irradiated for 20 min [UV20]) or control culture fluid. Actinomycin D or cycloheximide was added to cultures at the time indicated in column 1. At 5 hr pi, cells were examined for agglutination with 60 μg/ml of Con-A.

Fig. 3. Effect of actinomycin D and cycloheximide on concentrations of Con-A required to cause 4+ agglutination of cells inoculated with HSV. Cells inoculated with HSV irradiated for the periods indicated were incubated at 37°C for 6 hr with or without a metabolic inhibitor, actinomycin D (1 μg/ml) or cycloheximide (50 μg/ml) and examined for agglutination by Con-A. ○-○, HSV-inoculated cells without inhibitor; ●-●, HSV-inoculated cells with either actinomycin D or cycloheximide, both inhibitors brought about the identical patterns; ..., control cells with or without actinomycin D or cycloheximide.
with UV0-HSV were not agglutinated when either inhibitor was present in the cultures. However, both inhibitors did not affect the increase of agglutinability of cells exposed to UV 20-HSV. As also presented in Fig. 3, the appearance of cell agglutinability was inhibited by actinomycin D or cycloheximide only when the virus inoculum had been irradiated for a period of 5 min or less. Virus irradiated for periods longer than 7 min could induce agglutinability of cells in the presence of the inhibitor. Control cells similarly treated with the inhibitor were agglutinated only by 1,000 µg/ml of Con-A. These data suggest that there are two different phases of cell agglutination by Con-A; one requires de novo protein synthesis in HSV-inoculated cells and the other does not. These are tentatively called, herein, 1) “Early 1” or an inhibitor-sensitive agglutinability which is induced by active HSV or virus irradiated less than 5 min, and 2) “Early 2” or an inhibitor-insensitive agglutinability which is induced by HSV irradiated for a period longer than 7 min. It can be concluded from the results that “Early 1” and “Early 2” agglutinabilities are induced through two separate mechanisms by the inoculation of HSV.

Persistence of the Agglutinability

In order to determine how long the agglutinability of HSV-treated cells would persist, the following experiments were undertaken. Cells were inoculated with UV20-HSV at a moi of 5, incubated at 37 C and after appropriate intervals, examined for agglutination. “Early 2” agglutinability of cultures persisted for only 2 days, by which time it already had decreased markedly, and after 3 days these cultures did not differ from uninfected cultures in their agglutination properties (Fig. 4). “Early 1” agglutinability could not be tested for its persistence due to CPE developed in the cultures inoculated with HSV, either active or irradiated less than 5 min.
than 3 min, and very weak agglutinability of cells induced by virus irradiated for 4 or 5 min.

**Induction of “Late” Agglutinability**

Cells in suspension were exposed to UV6-HSV which was unable to produce “Early 1” and “Early 2” agglutinabilities. Plates were seeded at different cell concentrations. As shown in Fig. 5, agglutinability began to increase 3 days pi with UV6-HSV. Cells seeded at $5 \times 10^5$ cells/plate were regularly agglutinated by 62 $\mu$g/ml of Con-A at 5 and 6 days pi. Cells seeded at $1 \times 10^6$ were also agglutinated under similar conditions. UV6-HSV-exposed cells initially seeded at a higher cell concentration ($2 \times 10^6$ cells/plate, non-replicating cultures) were agglutinated only upon treatment with 1,000 $\mu$g/ml of Con-A. It should be noted that cells inoculated with UV6-HSV formed larger cell clumps upon treatment with 62 $\mu$g/ml of Con-A, while a portion of the cells still remained in single-cell suspension.

Cells were inoculated with HSV irradiated for various time periods. Cultures were examined for cell agglutination 6 days pi. Cells inoculated with virus which had been irradiated by UV for 4 to 10 min, were agglutinated by low concentrations of Con-A (Fig. 6), although a portion of cells in cultures exposed to UV4-HSV showed slight CPE. Cells exposed to virus irradiated for 15 or 20 min were agglutinated only by 500 or 1,000 $\mu$g/ml of Con-A, respectively. Control cells were agglutinated by 1,000 $\mu$g/ml of Con-A.

The agglutinability, observed 72 hr pi, has been described as “Late” agglutinability in order to differentiate this from “Early 1” or “Early 2” agglutinability. In replicate experiments, a significant increase of “Late” agglutinability could not be detected in non-replicating cultures inoculated with UV6- or UV8-HSV. Hence,
it appears likely that the replication of inoculated cells in cultures is required to increase the agglutinability with Con-A.

Morphological Transformation of 3T3 Cells Exposed to UV-Irradiated HSV

It has been reported that the transformation of cells treated with HSV was influenced by the length of time when the virus inoculum was exposed to UV (2, 8). Thus, it seems to be of interest to determine whether there is similar temporal pattern in two phenomena of agglutinability and transformation of the cells through the response of HSV to UV-irradiation.
3T3 cells in suspension were exposed to HSV which had been irradiated for periods of 4 to 10 min. The cells were seeded at $5 \times 10^4$ cells per plate, and incubated at 37°C. Frequency of the transformation of cells by the HSV used was expressed as a ratio of PFU/FFU (see Materials and Methods). Results are shown in Fig. 7. A high transformation rate was observed in cells exposed to HSV which had been irradiated for 6 to 8 min. Irradiation of virus for 7 min resulted in the maximal transformation rate of $7.1 \times 10^4$. The frequency of transformation of cells exposed to the virus irradiated for 4 or 10 min was less and irregular. However, there was no transformed focus in cell cultures exposed to control cell fluid, or to HSV, either active or irradiated for periods less than 3 min or more than 10 min. These results confirmed the data in previous reports (2, 8).

Cells were isolated from 10 transformed foci and cultured as established cell line, and cells from these lines were regularly agglutinable, without exception even at different cell passage levels, by Con-A at concentrations of 15 to 75 μg/ml. About 10 to 30% of cells from these lines contained HSV-specific viral antigen and HSV-neutralizing antibody was produced in mice after the inoculation of the transformed cells (data are not shown).

**DISCUSSION**

The results presented in this study indicate that 3T3 cells exposed to HSV showed an increase of agglutinability by Con-A, i.e., required low concentrations of Con-A to bring about a 4+ agglutination than did control cells. In addition to an agglutinability by Con-A of cells having been in contact with virus (infection time 0), HSV induced at least three different phases of cell agglutinability by Con-A. One was early-appearing agglutinability which began to increase 3 or 4 hr pi, and the other was late-appearing one which was detectable from 72 hr pi. The early one was further divided into two phases, based on its different sensitivity to actinomycin D (1 μg/ml) or cycloheximide (50 μg/ml). These were tentatively called “Early 1 or an inhibitor-sensitive”, “Early 2 or an inhibitor-insensitive” and “Late” agglutinability. The condition under which these occur are summarized in Table 2.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Appearance of agglutination</th>
<th>Early 1</th>
<th>Early 2</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial appearance</td>
<td></td>
<td>3 hr</td>
<td>4 hr</td>
<td>72 hr</td>
</tr>
<tr>
<td>Pattern of aggregates</td>
<td></td>
<td>soft</td>
<td>tight</td>
<td>soft</td>
</tr>
<tr>
<td>HSV irradiation (min)</td>
<td></td>
<td>0–5</td>
<td>7–20</td>
<td>4–15</td>
</tr>
<tr>
<td>Minimal Con-A conc. for 4+ agglutination</td>
<td></td>
<td>62 μg/ml</td>
<td>31 μg/ml</td>
<td>62 μg/ml</td>
</tr>
<tr>
<td>Cell replication</td>
<td></td>
<td>not required</td>
<td>required</td>
<td></td>
</tr>
<tr>
<td>Treatment with</td>
<td></td>
<td>Actinomycin D, 1 μg/ml</td>
<td>inhibited</td>
<td>not inhibited</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cycloheximide, 50 μg/ml</td>
<td>inhibited</td>
<td>not inhibited</td>
</tr>
</tbody>
</table>

NT, not tested.
It was previously reported that the agglutinability being insensitive to cycloheximide was detected in the cultures of 3T3 cells with UV-HSV (14).

The following criteria suggest that agglutination of 3T3 cells with Con-A was induced by HSV; 1) as described in a previous report (20), HSV treated with ether or heat did not induce agglutination, 2) after neutralization of HSV, both active and UV-irradiated, with anti-HSV immune serum, cell agglutination did not occur (Taguchi et al., unpublished data), 3) 3T3 cells which had been in contact with HSV for 90 min at 4°C were regularly agglutinated (Fig. 1), and 4) medium alone or extracts of control cells, either UV-irradiated or not, did not affect cell agglutination when added to cultures.

It has been reported previously that UV-irradiated HSV can activate an endogenous type C virus (9, 17). However, neither type C virus nor its antigen could be detected in cells transformed by UV-irradiated HSV (8). It appears unlikely that activation of a type C virus influenced agglutinability of 3T3 cells inoculated with irradiated HSV. This is because 1) as far as is known there is no direct evidence that agglutination of 3T3 cells with Con-A was due to any endogenous type C virus, and 2) “Early 1” and “Early 2” agglutinations were completely eliminated by UV-irradiation of virus for 6 min (Fig. 2).

Plausible explanations for the induction of agglutinability of the HSV-infected cells are as follows; 1) the agglutination of the cells at time 0 of virus infection was due to input virus itself, this is because there is evidence that HSV has specific receptors for Con-A (11, 15), 2) “Early 1” might be mediated by HSV-induced surface antigen which has been synthesized early in the infection and localized in cell membrane (10). A Con-A receptor was found in a viral glycoprotein possibly associated with cell membrane (16), 3) “Early 2” agglutinability might be due to certain components of the input virus; the hypothesis is based on its insensitivity to the metabolic inhibitors of cycloheximide and actinomycin D. Although the mechanism for “Early 2” induction is uncertain, it should be noted that there was a report which showed “cap” formation by exocytosis of the input-viral antigen of the cells inoculated with HVJ (12), and 4) “Late” one might be related to the expression of the viral antigen or the cell surface membrane modified by HSV. It should be noted, however, that by immunofluorescent test with HSV immune serum, viral antigen(s) could not be detected on the cell surface at 3 to 4 hr or at 72 hr pi (Taguchi et al., unpublished data), by which time three phases of agglutinability already had increased markedly.

It is worth noting that there were differential effects of duration of UV-irradiation of HSV on the increase of Con-A-mediated agglutinability and the rate of cell transformation. UV-inactivation of the infectivity of HSV appeared to affect inversely the induction of early-appearing cell agglutination and late-appearing one, or the frequency of cell transformation (Figs. 3 and 7). However, there was similar temporal pattern in the HSV response curves after UV-irradiation, the induction of “Late” agglutinability and the rate of cell transformation. As shown in Figs. 6 and 7, “Late” agglutinability, observed 72 hr pi, apparently increased in cultures treated with HSV which had been irradiated for 4 to 10 min. Among those, HSV irradiated
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for 6 and 8 min was most efficient. The frequency of transformation, observed 3 to 4 weeks pi, was also influenced by duration of time when HSV was subjected to UV-irradiation. A high transformation rate was observed in cell cultures exposed to HSV which had been irradiated for 6 to 8 min. These results indicate that the optimal duration of UV-irradiation of HSV inoculum seems to be limited to 6 to 8 min for two different phenomena. Any significance of the similarity is not known. The present results raise many questions regarding mechanisms for induction of the three different phases of the agglutinability of cells after exposure to UV-inactivated virus.

REFERENCES


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