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Effects of Lectins on Peripheral Infection by Herpes Simplex Virus of Rat Sensory Neurons in Culture

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Concanavalin A and wheat germ agglutinin are capable of preventing a productive peripheral infection of dissociated rat sensory neurons in culture by herpes simplex virus type 1. Concanavalin A binds to the herpes simplex virion, rendering it inactive, whereas wheat germ agglutinin binds to the peripheral neuritic extensions of the sensory neurons, rendering them incapable of initiating a productive viral infection. This latter effect (i) seems to be specific for wheat germ agglutinin since other lectins have no effect, (ii) is not the result of cellular cytotoxicity, (iii) is dependent on an *N*-acetylneuraminic acid moiety, and (iv) may be due either to viral receptor site masking or to binding of wheat germ agglutinin to the neuritic receptor molecule for herpes simplex virus.

Although the initial step in viral replication involves the interaction of virions with cell surface receptors, little basic science research has been conducted on this aspect of the viral replication process. The experimental procedure that has been used to study this interaction involved the incubation of virions or host cells with lectins, enzymes, or potential soluble or particulate receptor analogs, followed by a comparison with controls of the amounts of hemagglutination activity, plaque formation, uptake of radioactivity-labeled virus, or bound virus as determined by electron microscopy (3). Little is known about the receptors for herpes simplex virus (HSV). Attachment is promoted by thyroid hormone (18) and inhibited by parathyroid hormone (12). Concanavalin A (ConA) can bind to HSV virions and inactivate the virus (7, 10), but this inactivation may not involve the inhibition attachment of the virus to the cell (7). Cell surface receptors for HSV appear to be trypsin-sensitive glycoproteins with oligosaccharide chains (S. L. Lemaster and H. A. Blough, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S269, p. 249).

Since all of these studies involved the use of tissue culture cells which are not natural host cells primarily involved in HSV infection, we decided to study the interaction of HSV virions with sensory neurons, the natural site of latency of this virus. In addition, we decided to utilize a recently developed peripheral infection system (19) to obtain information which is perhaps more relevant to the natural HSV infection. In this study, we report the results of our studies using this system and various lectins to obtain preliminary information about the nature or identity of the HSV virion receptors and neuronal cell surface receptor sites for HSV.

MATERIALS AND METHODS

Virus. HSV type 1 (HSV-1; MacIntyre strain) was grown in HEp-2 monolayers and harvested 48 to 72 h after infection by freeze-thawing followed by low-speed centrifugation to remove cellular debris. The virus suspension was rapidly frozen in a dry ice-95% ethanol bath and stored at −70°C. The titer of the virus preparation was determined by plaque formation in BHK-21 cells by using standard techniques.

Dissociated sensory neuron cultures. Cultures of dissociated sensory dorsal root ganglia neurons were prepared from 17-day fetuses as previously described, with the exception of two minor modifications (19). In this case, the cells used for these studies were obtained without teasing the ganglia, and trypsin treatment was employed for only 30 min.

The culture system involved placing dissociated dorsal root ganglia cells inside a cloning cylinder, which was sealed to a tissue culture plate with vacuum grease. After 10 to 14 days of culture, neurites began to enter the area outside the cloning cylinder. Cultures at this stage of growth were used for peripheral infections.

Peripheral infection of sensory neuron cultures by HSV. The medium was removed from the area outside the cloning cylinder. After two washes with Hanks balanced salt solution (HBSS) (pH 7.2), 10^3 plaque-forming units (PFU) of HSV in 1.0 ml of HBSS was applied to this area. The cultures were rocked for 60 min at 37°C, the virus suspension was removed, and 1.0 ml of Eagle medium (pH 7.2), containing 10% fetal calf serum and 10% horse serum (K. C. Biological, Inc., Lenexa, Kans.), was added. These infected plates were then incubated at 37°C and

viewed by phase-contrast microscopy for cytopathology at 24, 48, or 72 h.

Alternatively, sample cultures were taken at selected time intervals (usually 48 h postinfection [p.i.]) and examined for the presence of HSV antigens. The areas inside and outside the cloning cylinder were washed with 0.01 M phosphate-buffered saline (pH 7.2), fixed in 95% ethanol, and stained with fluorescein-labeled rabbit anti-HSV antibody (M. A. Bioproducts, Walkersville, Md.) to detect the presence of HSV-specific antigens in cells.

Lectin treatment of HSV and dissociated sensory neuron cultures. A 0.1-ml sample (10^5 PFU) of HSV was added to 0.9 ml of a 100-μg/ml solution of lectin (E-Y Laboratories, Inc., San Mateo, Calif.) in HBSS. After 15 min of incubation with gentle rocking at 37°C, 0.1 ml of a 1:10 dilution of the virus-lectin mixture (10^3 PFU of HSV and 1 μg of lectin) was added to a tube containing 0.4 ml of HBSS. The entire contents of this tube were then used to peripherally infect the sensory neuron cultures. For plaque neutralization assays 0.1 ml of the virus-lectin mixture was diluted with 9.9 ml of HBSS and titrated on BHK-21 cells.

In cultures that were to be treated with lectin before exposure to HSV, the area outside the cloning cylinder was first rinsed twice with HBSS. A 0.5-ml volume of HBSS containing 100 μg of lectins was added to this area, and the culture plate was rocked gently for 15 min at 37°C. The lectin solution was removed, and the cultures were rinsed twice with HBSS before infection with 10^3 PFU of HSV.

HSV neutralization by carbohydrates. A 0.1-ml sample (10^3 PFU) of HSV in HBSS was added to 1.0 ml of HBSS containing a 0.3 M solution of either *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, or *N*-acetylneuraminic acid (NeuNAc; Sigma Chemical Co., St. Louis, Mo.), or a 0.03 M solution of *N,N'*-diacetylchitobiose (E-Y Laboratories). All lectin solutions were prepared in HBSS. After 15 min of incubation with gentle rocking at 37°C, 0.7 ml of the virus-carbohydrate mixture was added to appropriate cultures to initiate peripheral infections, and 0.2 ml was titrated on BHK-21 cells.

RESULTS

Time sequence of peripheral infection. We have previously described an in vitro peripheral infection system of sensory neurons by HSV-1 (19). Figure 1 illustrates the time sequence of HSV-1 antigen production within neuronal somas and adjacent cells after peripheral infection by the virus. At 16 h p.i., all cells outside the cloning cylinder were rounded and showed HSV-1 antigens as indicated by HSV-1-specific fluorescent-antibody staining. In addition, some peripherally infected neuronal somas also showed HSV-1 antigen synthesis (Fig. 1A). By 24 h p.i., the virus infection spread to neighboring neurons, satellite cells, and Schwann cells (Fig. 1B). The infection continued to spread, involving increased numbers of cells, at 48 h p.i. (Fig. 1C). At 72 h p.i. almost all cells within the cultures were producing viral antigens (Fig. 1D). This time sequence was consistent throughout a number of trials. As a result, for the remaining studies in this report we chose 48 h p.i. as the time at which to stain with fluorescent antibody to determine whether a productive peripheral infection of neurons had occurred.

HSV neutralization by lectins. ConA has previously been reported to inactivate the infectivity of HSV for FL cells (10) and BGM cells (7). It is not known whether this lectin or other lectins can abolish the infectivity of HSV-1 for sensory neurons by either direct or peripheral infection. Table 1 presents results which indicate that ConA can indeed inactivate the infectivity of HSV-1 for peripheral infection of sensory neurons, and that under the experimental conditions used it can cause almost a 100-fold reduction of virus titer. Other lectins utilized showed little effect on virus titer and no effect on peripheral infection of sensory neurons.

Inhibition of HSV infection by lectin treatment of peripheral neurites. The chemical nature of the HSV-1 binding sites on sensory neurons and, more specifically, on peripheral neurites, where the initiation of natural infection occurs, is not known. The data presented in Table 2 represent an attempt to learn about this binding site by using lectins as a probe. Most of the lectins used have different carbohydrate specificities, although some overlapping of specificities does occur. Wheat germ agglutinin (WGA) and *Ricinus communis* agglutinin (RCA) consistently displayed an ability to inhibit peripheral infection by the virus. Other lectins were consistently negative.

Lectin binding and toxicity to cell cultures. Since RCA and WGA have been shown to be toxic to some cells, it became important to determine whether the inhibition of HSV-1 infection reported in Table 2 was due to cellular cytotoxicity or some more specific effect of these lectins. The data presented in Table 3 indicate that the inhibition by RCA was probably due to cellular cytotoxicity. The peripheral area of the cultures was exposed to either fluorescein-labeled or unlabeled lectins under normal experimental conditions, and the cultures were examined 15 min and 24 and 48 h later for signs of cytopathology.

Fluorescein-labeled RCA and WGA studies indicated that initially both lectins bound to the neurites and the periphery of Schwann cells, but by 24 h p.i. the lectin had disappeared from the neurites and became internalized about the nucleus of Schwann cells (Fig. 2; only WGA data

A
B
C
D

TABLE 1. *HSV neutralization by lectins*[a]

Lectin[b]	PFU on BHK-21 cells	HSV peripheral infection
None	3.1×10^4	+
ConA	5.0×10^2	−
WGA	1.2×10^4	+
LPA	2.3×10^4	+
SBA	3.4×10^4	+
UEA	2.6×10^4	+
DBA	3.0×10^4	+

[a] A 100-μg sample of each lectin was incubated with 10^5 PFU of HSV for 15 min at 37°C, and a 1:100 dilution of this mixture was used to peripherally infect cell cultures. At 48 h p.i., cultures were stained for HSV-specific immunofluorescence. The amount of fluorescence determined the extent of infection and was compared with that in untreated HSV-infected cultures. Samples of the virus-lectin mixture were titrated on BHK-21 cells and read 24 h p.i.

[b] Abbreviations: LPA, *L. polyphemus* agglutinin; SBA, *Glycine max* agglutinin; UEA, *Ulex europaeus* agglutinin; DBA, *Dolichos biflorus* agglutinin.

TABLE 2. *Extent of HSV infection after pretreatment of peripheral neurites with lectins*[a]

Lectin (source)	HSV infection
None	+
WGA (*Triticum vulgaris*)	−
RCA (*Ricinus communis* L.)	−
STA (*Solanum tuberosum*)	+
ConA (*Canavalia ensiformis*)	+
LPA (*Limulus polyphemus*)	+
SBA (*Glycine max*)	+
UEA (*Ulex curopaeus*)	+
DBA (*Dolichos biflorus*)	+

[a] The areas of the cultures containing the peripheral neurites were incubated with 100 μg of lectins for 15 min at 37°C before exposure to 10^3 PFU of HSV. At 48 h p.i., cultures were stained for HSV-specific immunofluorescence. The amount of fluorescence determined the extent of infection and was compared with that in untreated cultures infected with HSV.

shown). Peripheral cells at this time appeared normal in WGA-treated cultures and were rounded in RCA-treated cultures. By 48 h p.i. there was no change; the distribution of fluorescein-labeled WGA and the cells still appeared normal. However, the RCA-treated cultures were devoid of peripheral cells and neurites.

TABLE 3. *Toxicity of lectins to neurons after peripheral exposure*

Lectin	Cellular cytopathology[a]	HSV infection[b]
None	−	+
WGA	−	−
RCA	+	−

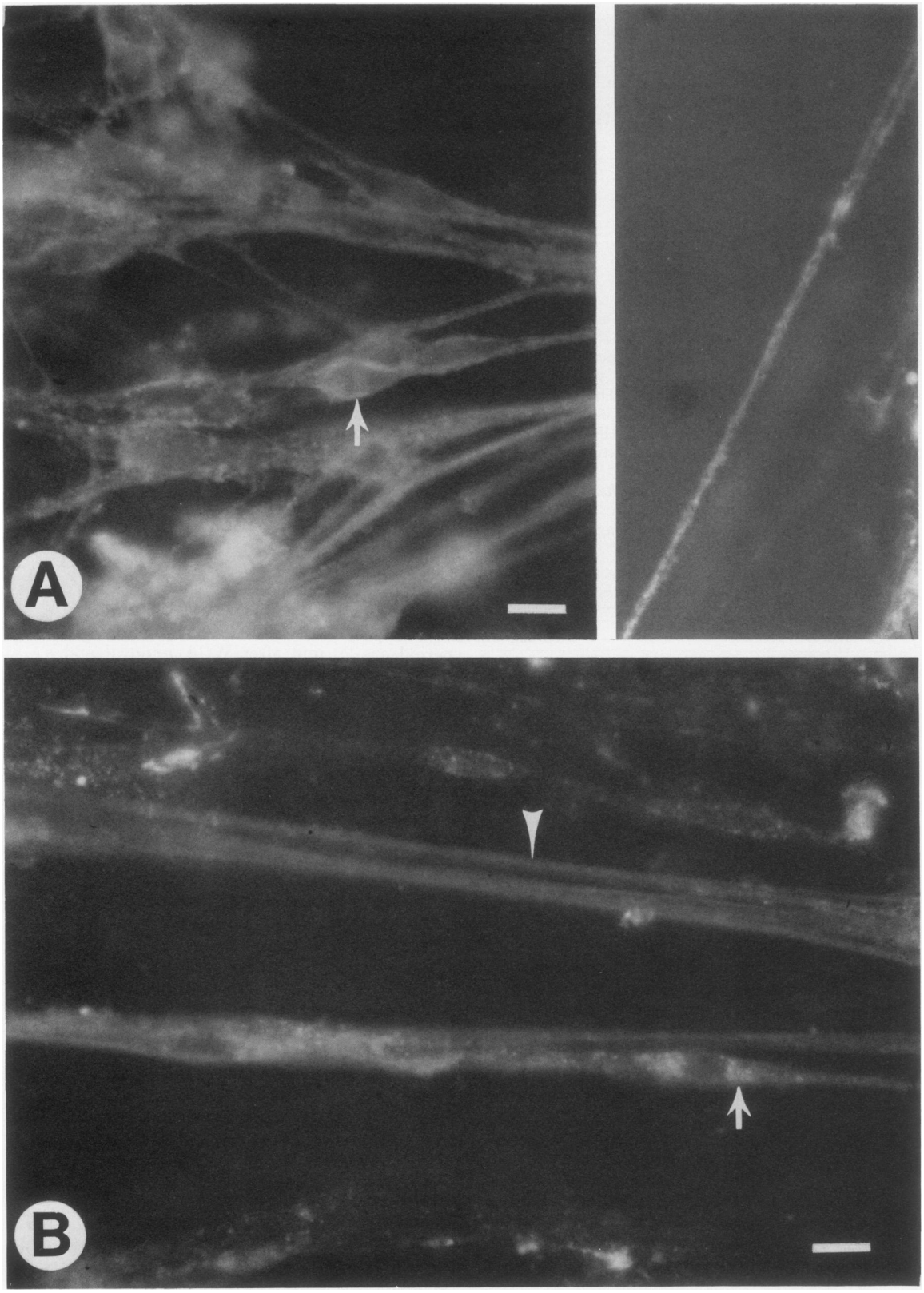
[a] The areas of the cultures containing peripheral neurites were incubated with 100 μg of lectin for 15 min at 37°C. The lectin solution was removed and replaced with growth medium. The cells in this area were examined immediately and at 24 and 48 h by phase-contrast microscopy for cytopathology.

[b] Analysis and quantitation were performed as described in Table 2.

Reappearance of HSV infectivity after WGA treatment. The data in Table 4 confirm the suggestion that WGA does not inhibit HSV peripheral infection by killing the host cells. These data indicate that 80 min after the removal of WGA the culture was susceptible to HSV peripheral infection. The infection was not as widespread as is normally seen in these cultures at this time. (Included in this 80-min time period are 20 min after WGA removal and a subsequent 60-min viral adsorption period.) By 120 min the normal amount of viral infection appeared in the cultures. Clearly, if WGA killed the neurons, productive infection could not occur. The inhibition of HSV peripheral infection by WGA might, therefore, be due to a more specific mechanism such as the binding of WGA to viral receptors, rendering them unavailable to the virus.

HSV neutralization by carbohydrates. If WGA inhibits HSV peripheral infection by binding to viral receptors, then perhaps preincubating HSV with the carbohydrates known to bind to WGA might indicate which of these compounds is important for HSV attachment to the neurite. Table 5 contains the results of these experiments. Preincubation of HSV with *N*-acetyl-D-glucosamine, *N,N'*-diacetylchitobiose (β-D-[1,4] dimer of *N*-acetyl-D-glucosamine), and *N*-acetyl-D-galactosamine did not neutralize the virus's ability to cause a peripheral infection or

FIG. 1. *Immunofluorescent micrographs of rat neuronal somas inside the cloning cylinder at various times post-peripheral infection with HSV. (A) Group of neuronal somas at 16 h p.i. Only an occasional soma has HSV-specific fluorescence. (B) Photograph taken at 24 h p.i. shows a cluster of somas in which four somas contain HSV-specific fluorescence. Several of these are seen within each culture at this time. In addition, one can see that the virus has also begun to spread to the Schwann cells, since several of these (arrowheads) also show fluorescence. (C) and (D) These photographs—(C) at 48 h p.i. and (D) 72 h p.i.—show the progressive involvement of additional neuronal somas and Schwann cells in HSV replication until finally all cells display HSV-specific fluorescence. Small bar represents 30 μm and large bar represents 60 μm.*


A
B

significantly lower viral titer. In the three experiments involving preincubation with NeuNAc, viral titer dropped over 90% and no peripheral infection was observed. This result suggests that the membrane receptor for HSV is a molecule that contains a NeuNAc moiety.

Neuraminidase (type V from *Clostridium perfringens*; Sigma) treatment of neurites before exposure to HSV confirmed this finding. Cultures whose peripheral neurites were incubated with 5.0 U of neuraminidase for 60 min at 37°C before exposure to HSV showed no signs of a productive peripheral infection and did not bind fluorescein-labeled WGA. Pretreatment with 1.0 U or less of neuraminidase displayed a reduced or normal level of peripheral infection.

DISCUSSION

These experiments were initiated to study the chemical nature of HSV receptor and sensory neuron receptor sites necessary for the initiation of peripheral infection of these neurons by HSV. The strategy employed was to utilize lectins with different carbohydrate binding specificities as probes for determining the possible importance of specific carbohydrate moieties for effective virus-cell interaction, and perhaps to identify a specific cellular carbohydrate moiety as being essential for the absorption of the virus. This latter formation would then be used as a basis for further studies to determine the precise neuronal glycoprotein or glycolipid necessary for initiating peripheral HSV infection.

TABLE 4. *Reappearance of HSV infectivity after removal of WGA*[a]

Time (min) after WGA removal	HSV peripheral infection
60	−
80	+
120	+

[a] A 100-μg sample of WGA was applied for 15 min at 37°C to the peripheral neurite area of three cultures. After this application, one culture (60 min) was exposed immediately to 10^3 PFU of HSV for 60 min. Another culture (80 min) was incubated in growth medium for 20 min at 37°C before infection, and the third culture (120 min) was incubated for 60 min at 37°C in growth medium before infection. All cultures were examined at 48 h p.i. as described in Table 2 for the extent of HSV infection.

TABLE 5. *HSV neutralization by carbohydrates*[a]

Carbohydrate	PFU on BHK-21 cells	HSV peripheral infection
None	7.8×10^2	+
N-Acetyl-D-glucosamine	7.9×10^2	+
N,N'-Diacetylchitobiose	7.7×10^2	+
N-Acetyl-D-galactosamine	7.6×10^2	+
NeuNAc (sialic acid)	7.4×10^1	−

[a] To 0.1 ml (10^3 PFU) of HSV was added 1.0 ml of a 0.3 M solution of either *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, or NeuNAc or a 0.03 M solution of *N,N'*-diacetylchitobiose, and each virus-carbohydrate mixture was incubated for 15 min at 37°C. A 0.2-ml sample of the virus-carbohydrate mixture was titrated on BHK-21 cells and read at 24 h p.i. Volumes of 0.7 ml of each mixture were used to peripherally infect cell cultures, and the extent of HSV infection was determined as described in Table 2.

The data presented in Table 1 illustrate that ConA can react with HSV virions and render them nonproductive for peripheral infection of sensory neurons in culture as well as lower viral titers when assayed on BHK-21 cells. This inactivation of HSV virions by ConA is not a new finding (7, 10), but adds to the types of cells and types of infection systems which are known to be nonproductive when exposed to ConA-treated HSV. Whether ConA-treated HSV does not attach to peripheral neurites or does not penetrate has not been resolved. Immunofluorescent staining of neurites immediately after exposure to ConA-treated HSV showed no staining (unpublished data), but this could be the result of several possibilities, including masked antigens, insufficient concentration of antigens for visible staining, etc., rather than lack of attachment.

The data in this report concerning the nature of the neuronal receptor site for HSV suggest that *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, or NeuNAc may be part of that receptor, since WGA binds to the surface of neurites and inhibits peripheral infection by HSV. This inhibition is not due to cellular toxicity, as observed with other lectins which bind to the neurite, like RCA. The inhibition is apparently spe-

FIG. 2. *Fluorescent photomicrographs of peripheral cells and neurites exposed to fluorescein-labeled WGA. (A) Specific diffuse fluorescent staining of neurites and the periphery of cells (arrow) after treatment with 100 μg of fluorescein-labeled WGA for 15 min at 37°C. The inset contains an isolated neurite magnified an additional 2.5 times. (B) Cultures treated as in (A), but incubated after lectin solution removal for an additional 24 h at 37°C. The peripheral labeling of cells seen in (A) has progressed to a granular intracytoplasmic fluorescence (arrow), and the fluorescence indicating WGA binding to neurites has disappeared (arrowhead). Bars represent 20 μm.*

cific since other lectins like fluorescein-labeled ConA also bind, but do not inhibit.

The inhibition of HSV peripheral infection and an over 90% reduction in viral titers after preincubation of the virus with NeuNAc suggests that NeuNAc is a component of the cellular receptor. For sensory neurons, this hypothesis is supported by the observation that pretreatment of neurites with 5 U of *C. perfringens* neuraminidase renders the culture incapable of producing a productive infection. The *Limulus polyphemus* agglutinin lectin also has binding specificity for NeuNAc residues, but does not inhibit, suggesting that the receptor may be composed of additional structural components recognized by WGA but not by *L. polyphemus* agglutinin. This result is not surprising since receptor sites for other virus have been shown to be glycoproteins or gangliosides (2, 3, 5, 6, 8, 14, 18), and in fact, the cell surface receptor site for HSV on BHK-21 cells has been reported to be a trypsin-sensitive glycoprotein (Lemaster and Blough, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S269, p. 249).

Our inhibition of HSV peripheral infection of sensory neurons by WGA can be interpreted in two ways. The first interpretation is that the inhibition by WGA is simply due to a masking phenomenon. WGA is a dimeric protein with a molecular weight of 35,000 (3, 9, 11) which contains several carbohydrate binding sites (16, 17) and binds to a variety of cellular sialoglycoproteins (1) and glycosphingolipids which possess NeuNAc moieties (15). Perhaps WGA binds to a membrane glycoprotein or glycolipid which is not the true viral receptor site but is located near the true site and sterically hinders effective receptor-receptor site interaction. This hypothesis may be supported by the rapid reappearance of receptor sites which allow productive infection after WGA is removed from contact with neurites (20 min; see Table 4). This short time interval may preclude new receptor site synthesis.

The other interpretation would be that the neuronal receptor site is a molecule which contains NeuNAc and can bind WGA, but not *L. polyphemus* agglutinin. The topography of NeuNAc and *N*-acetyl-D-glucosamine residues on glycoconjugates and the cell surface has been shown to be important for stable WGA interaction (1). Perhaps the differential binding of WGA and *L. polyphemus* agglutinin to various membrane molecules containing NeuNAc residue will aid the identification of the cellular HSV receptor molecule.

We are beginning to study the ability of more complex biochemical compounds such as glycoprotein and gangliosides to neutralize HSV virion infectivity. These experiments, as well as similar studies using glycoproteins or glycolipid obtained from sensory neuronal membrane and capable of binding to WGA-agarose beads, should be helpful in identification of the neuronal membrane receptor molecules for HSV.

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