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The Nature of Toxins of Herpes Simplex Virus

I. Syncytial Giant Cell Producing Components in Tissue Culture[1]

By

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With 8 Figures

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The virus of herpes simplex is known to produce rather infrequent toxic manifestations. The diversity of such toxic manifestations has long been a subject of some interest. The severity of these toxic effects when manifested is only equalled by that of the more potent bacterial toxins. None of the toxic components of the herpes simplex virus has been isolated, and no methods to prevent their effects are known.

We previously reported the occurrence of a high fever after the intravenous injection of large amounts of this virus in guinea pigs and rabbits (1). The febrile pattern was comparable to that produced by influenza virus and other members of the myxovirus group.

The objectives of the study reported here were the isolation of herpesvirus pyrogens which are specifically antigenic, their physicochemical characterization, and the determination ot their relationship to the experimentally reproducible toxicity of this virus. The experimental procedures for studying the viral toxins in general have previously been reviewed (2—5).

Some of the myxoviruses contain hemolysins. Such viruses as those of mumps (6—7), parainfluenza (8) and measles (9, 10) also have been shown to produce cytolysis or to have injurious effects on cell membrane resulting in the formation of syncytial giant cells.

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In our earlier reports (11—13) and others (14) it was reported that certain strains of herpesvirus were also capable of producing such syncytia. It further was reported that such syncytia could be produced by herpesvirus which had been partially inactivated by ultraviolet irradiation (15).

Further investigation of this effect of herpes simplex virus provided evidence that it was caused by a viral component which appeared to be lipoprotein in nature. The lability of this component appeared to correspond to that of some previously studied labile pyrogen.

Materials and Methods

Preparation of herpes antigens. The H strain of herpes simplex virus was propagated in primary cultures of rabbit kidney cells and the antigens were prepared as previously described (16, 17) with the following important difference: It was shown in pyrogenicity studies (1) that at least 10^9 plaque-forming units (p.f.u.) per ml are required to produce an adequately demonstrable toxicity. For this reason the virus was concentrated from the original culture fluid, which contained $10^{7.5}$ p.f.u. per ml, by ultracentrifugation. In addition, some pyrogens which could be neutralized by specific antiserum also were present in the supernatant fluid after sedimentation of the virus. By selecting good passage virus of the H strain during 12 passages it proved possible to obtain $10^{9.3}$ p.f.u. per ml of tissue culture fluid. The seed virus was inoculated in a virus/cell ratio of 1:5 and the inoculated culture was then incubated at 37°C for 24 hours. The culture fluid was harvested at the time of maximal syncytium formation, shortly before the sloughing off of the cell sheet from the glass. The culture fluid was centrifuged at 2500 r.p.m. for 30 minutes and the supernatant fluid was titrated for infectivity and pyrogenicity. It showed a pyrogenicity equal to that of concentrated virus. We shall refer to this material as crude herpesvirus.

Observation of cytopathic effects. Cultures of rabbit kidney cells were obtained by seeding 10^6 cells per ml after trypsinization into ordinary glass tissue culture tubes, which were refed with 1 ml amounts of medium on the 3rd and 5th days after the seeding. On the 5th day of incubation, when each tube contained a full sheet of ca. 10^6 cells, these cell cultures were inoculated with 1 ml amounts of the crude virus and the cultures were subsequently examined at various times for the formation of syncytia or degeneration as evidenced by rounding of the cells (Fig. 7). These cytopathic changes were essentially the same as those previously reported for the L and G strains of pseudorabies virus (11) and for the GC and P strains of herpes simplex virus (12, 13). The culture tubes were incubated at 37°C unless otherwise specified. Dilution of the crude virus were made with the tissue culture medium used for the cultures of rabbit kidney cells.

Neutralization studies. Immune serum globulin (human gamma globulin) (Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York) which had a neutralizing antibody titer for herpesvirus of 1:2000 was diluted 20-fold in tissue culture medium. It was added in 0.2 ml amounts to 1 ml of the crude virus, and incubated at 37°C for 60 minutes before the titration in tissue culture tubes for the syncytium producing activity. Rabbit antisera were prepared by producing herpetic kerato-conjunctivitis in the

animals (by inoculation of the virus onto the scarified cornea) and collection of the serum was made 1 month later. The rabbit serum was inactivated at 56°C for 30 minutes before use.

Ultrafiltration. The materials were concentrated in a Diaflo Model 400 apparatus using a UM-1 membrane (Amicon Corporation, 25 Hartwell Ave., Lexington, Massachusetts) for 60 minutes. Small amounts were concentrated by negative pressure dialysis. To insure bacterial sterility the materials were filtered through a Nalgene membrane (0.45 micron grid) (The Nalge Co., Rochester, New York) when such sterility was required.

Gel filtration. The method used was that previously described for Sephadex G-200 (17). Biogel A-50m (Bio-rad Laboratories, 32nd and Griffin Sts., Richmond, California) was also used in columns of the same size and filtration was carried out in the same manner.

Density gradient centrifugation. Sucrose density gradients were established in 5 ml lusteroid tubes (type used in the SW 39 rotor of a Spinco Model L ultracentrifuge) with 0.7 ml layers of 45, 37, 29, 20 and 11% sucrose solutions, respectively, and 0.5 ml of 3 times concentrated crude virus was layered on top. The gradient tubes prepared in this manner were centrifuged at 30,000r.p.m. for 2 hours. Drops were then collected as previously described (17).

Freezing and thawing procedure. The crude virus in amounts of 2 ml and 5 ml were placed in Wassermann tubes which were closed with rubber stoppers. The contents of the tubes were frozen in a dry ice-alcohol bath and thawing was carried out at 37°C. Those tubes containing 2 ml were frozen by the shell-freezing method.

Ultraviolet irradiation. The crude virus was irradiated with ultraviolet light as previously described (13).

Enzymatic digestion. Two-fold serial dilutions of the enzymes were prepared in 0.2 ml amounts and mixed with 1 ml of crude virus and incubated at 37°C for 60 minutes. The preparations were then titrated for cytopathic activity in tube cultures.

Subjection of crude virus to various pH values. Fifteen ml portions of the crude virus were adjusted to the desired pH values with 0.2 *N* HCl or 0.2 *N* NaOH, allowed to stand for 60 minutes at room temperature, and then neutralized. Two-fold serial dilutions of the neutralized preparations were then made and 1 ml portions of the dilutions were added to tube cultures of the kidney cells.

Histological observations. Cells were grown in tissue cultures with small coverslips. At the usual harvest time the coverslips were removed and stained for histological studies. For staining of acid phosphatases Kit 85A-2 (Sigma Chemical Co., 3500 DeKalb St., St. Louis, Missouri) was used according to the manufacturer's instructions. Rabbit anti-herpes sera obtained as described above were labeled with fluorescein and used for direct detection of herpes virus antigens by fluorescent antibody staining.

Studies with leukocytes. Guinea pigs and mice were inoculated intraperitoneally with 3 ml and 0.5 ml of mineral oil, respectively, 24 hours before harvesting of the leukocytes. The animals were sacrificed by section of carotid. The peritoneal exudate was washed out with tissue culture medium and the leukocytes were collected by centrifugation at 1500 r.p.m. for 15 minutes. A suspension of 10^6 cells/ml was prepared and mixed with an equal amount of crude virus. The mixture was incubated at 37°C. Tenth-ml samples were

removed at various times and stained with 10 volumes of a 1% solution of Evans blue (Fisher Scientific Co., Fair Lawn, New Jersey) in physiological saline. The numbers of living (unstained) cells and the dead (blue-stained) cells were counted in an ordinary hemocytometer.

Sources of biochemical and chemical preparations used. The trypsin (bovine pancreas) Type I (2× crystallized), lecithinase-C *(Clostridium welchii)* Type I, lecithinase-D (Phospholipase) (cabbage) Type II, gamma-chymotrypsin (bovine pancreas) Type II 2× crystallized, ficin (fig tree latex) and lipase (wheat germ) Type I were all obtained from the Sigma Chemical Co. The acid phosphatase (wheat germ), purified alkaline phosphatase (calf intestine), lyophilized deoxyribonuclease I (bovine pancreas) and ribonuclease (bovine pancreas, 2× crystallized) were obtained from the Mann Research Laboratories, New York 6, New York. The trypsin inhibitor (soybeans), 5× crystallized, was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, the heparin (Liquaemin) from Organon, Inc. Orange, New Jersey, Lecithin RG from Amend Drug & Chemical Co., Inc., 117 East 24th St., New York 10, New York, the 5-iodo-2′-deoxyuridine (Stoxil) (IUDR) as a gift from Smith, Kline and French Laboratories, Philadelphia, Pennsylvania, and oxytetracycline (terramycin) from Chase, Pfizer and Co., Inc., New York.

Tissue culture cell lines. These were kindly provided by Dr. *G. Henle* of this institution.

Results

The Rate of Syncytium Formation

The earliest syncytium formation was observed in the kidney cell cultures 70 minutes after the inoculation of the crude virus, after which it progressively spread until it involved the whole cell sheet within 3 hours. When various dilutions of the crude virus were inoculated the time of the first appearance of the syncytium formation was delayed in direct proportion to the dilution of the virus, as is shown in Fig. 1. We graded the amount of syncytium-producing activity by assigning a value of 1 unit to that amount of activity which produced syncytium formation in 25% of a cell culture after incubation at 37°C for 7 hours. The reciprocal of the highest dilution producing this effect was considered to be the number of units contained in 1 ml of the undiluted crude virus. The activities of various batches of the crude virus varied from 8 to 64 units per ml. Since these crude virus preparations ordinarily contained $10^{9.3}$ p.f.u./ml of infectious virus, 2000 p.f.u. of virus per kidney cell were required to produce such an effect within 4 hours. If the average syncytium-producing activity in crude virus is assumed to be 32 units per ml, virus: kidney cell ratio for 1 unit of syncytium-producing activity was approximately 30:1.

Effect of Incubation Temperature on the Rate of Syncytium Formation

The rate of syncytium formation was temperature-dependent. At 4°C there was no syncytium formation even after 24—48 hours. However, preparations which had thus been kept at 4°C showed no loss in the

syncytium-producing activity when they were brought to a temperature of 37°C, indicating a persistent firm association of the syncytium-producing component with the cellular elements. At temperature of 31°C and 26°C (Fig. 1) the rates of syncytium formation were proportionally slower. At 26°C (room temperature), a syncytium was produced by 16 or more syncytium-producing units only after 18 hours. Hemolysis by mumps virus is known to proceed even at 26°C (10).

Production of Syncytium-producing Components

Tenfold serial dilutions of crude virus, 1:1, 1:10, 1:100 and 1:1000, respectively, were inoculated in 9 sets of tube cultures in 1 ml amounts. They were incubated at 37°C for the assigned period of time as indicated at the left-hand column in Table 1. Each set of tubes was then removed from an incubator and placed at 26°C, at which temperature herpesvirus

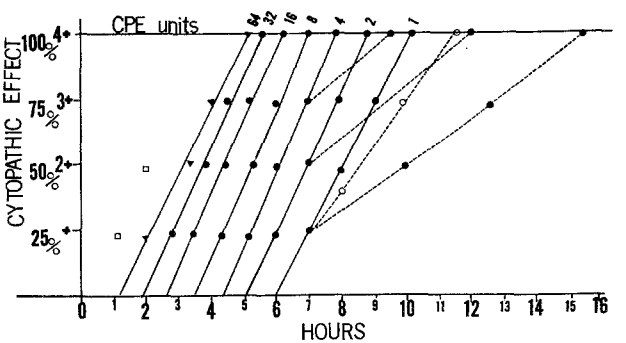

Fig. 1. The rate of syncytium formation. Incubation temperature at 37°C (●———●), 31°C (○-----○) and 26°C (●-----●). For CPE units see text. The time of some early degenerative rounding is shown in (□).

did not multiply (18). Eighteen hours after the last set was taken out and left at 26°C, all tubes were examined for syncytium formation (see Table 1). All tubes were then incubated at 37°C again for 5 hours. During this incubation, syncytium developed rapidly in the tubes which had received the crude virus dilutions of 1:10 and 1:100, especially in the sets which were originally incubated for 2 hours or more at 37°C, whereas syncytium formation was significantly delayed or not produced in those tubes which had received the same amounts of virus but were incubated at 37°C originally for 1 hour 30 minutes or less. We interpreted this result as indicating that a new synthesis of syncytium-producing components began at about 1 hour 45 minutes after the original inoculation of crude virus and its dilutions. These components were further synthesized during incubation at 37°C. Thus the amounts of syncytium-producing components were as much as 1024 units per ml of packed cells, which were

Table 1. The Effect of Shifting the Incubation Temperature on Syncytium Production

Time of the first incubation at 37° C	Cytopathic effects at the end of the first incubation		Time of the second incubation at 37° C (hours)			
	Virus dilutions	18 hours at 26° C	2	3	4	5
0 hour	1[1]	0 0—+	+	+	+	++
	1:10	0 0	0	0	0	+
	1:100	0 0	0	0	0	0
	1:1000	0 0	0	0	0	0
1 hour	1	0 0—+	+	+	+	++
	1:10	0 0	0	0	0	0
	1:100	0 0	0	0	0	0
	1:1000	0 0	0	0	0	0
1 hour 30 minutes	1	0 0—+	+	+	+	++
	1:10	0 0	0	0	0	+
	1:100	0 0	0	0	0	0
	1:1000	0 0	0	0	0	0
1 hour 45 minutes	1	0 +	++	+++	+++	++++
	1:10	0 0	+	++	++	+++
	1:100	0 0	0	0	0	+
	1:1000	0 0	0	0	0	0
2 hours	1	0 +	++	+++	+++	++++
	1:10	0 0	+	++	++	+++
	1:100	0 0	+	+	+	++
	1:1000	0 0	0	0	0	0
2 hours 30 minutes	1	0 +	+++	++++	++++	++++
	1:10	0 0	+	++	+++	++++
	1:100	0 0	+	++	++	+++
	1:1000	0 0	0	0	0	0
3 hours	1	0 ++	++++	++++	++++	++++
	1:10	0 ++	+++	+++	++++	++++
	1:100	0 0	+	++	++	++
	1:1000	0 0	0	0	0	0
4 hours	1	0 ++++	++++	++++	++++	++++
	1:10	0 ++	+++	++++	++++	++++
	1:100	0 +	+	+	++	+++
	1:1000	0 0	0	0	+	++
5 hours	1	+ ++++	++++	++++	++++	++++
	1:10	0 +++	+++	++++	++++	++++
	1:100	0 +	++	++	+++	+++
	1:1000	0 0	+	+	++	++

[1] Virus p.f.u. $10^{9.3}$ ml. The number of cells = 10^6 per culture.

first harvested from 32-oz. bottle cultures at the time of maximal cytopathic effect after the inoculation of low multiplicity of virus per cell and packed by centrifugation at 2000 r.p.m. for 15 minutes and then disrupted by ultrasonication.

Suppression of Cellular Activities

Production of both infectious virus and syncytium-producing components was suppressed when large amounts of the latter (16 units or more/ml) were initially added to the culture, as was reported earlier from this laboratory (12). The syncytial giant cells thus produced were not strongly stained with fluorescent antibody.

The formation of syncytium itself was not inhibited by the presence of IUDR in the medium at a concentration of 200 μg/ml which does

Table 2. Correlation between Herpesvirus Neutralizing Antibody Titers and Ability to Neutralize Syncytium-producing Components in Human Sera

Serum No.	Virus neutralizing antibody titers	Syncytium-producing components neutralizing antibody titers
1	16	16
2	8	8
3	16	16
4	16	16
5	<4	<4
6	128	32
7	16	4
8	<4	<4
9	<4	<4
10	128	128

inhibit virus multiplication (19). On the other hand, 10—90% of syncytium formation was prevented when 200 μg/ml of hydrocortisone was incorporated in the culture medium.

Immunological Specificity of Syncytium-producing Components

No syncytium was formed after preliminary incubation of the syncytium-producing components with human convalescent phase anti-herpes sera, but no such inactivation was produced by the acute phase sera from human patients with herpes infections. The neutralizing titers for the syncytium-producing components approximated the virus neutralizing antibody titers (Table 2). Human gamma-globulin and rabbit antiherpes hyperimmune sera also showed such neutralizing activity against syncytium-producing components. Sera were diluted twofold in 0.2 ml amounts and 1 ml of crude virus containing 8 units were mixed

and incubated at 37°C for 60 minutes. The end point of neutralization was read at 6 hours after above mixture was placed in the kidney cell tubes.

Adsorption of the Syncytium-producing Components on Kidney Cells

Whereas 90% of a suspension of infectious herpesvirus was adsorbed on cell sheets after incubation for 90 minutes, only 20% of the syncytium producing activity was adsorbed in this time. When the cultures on which this material had been adsorbed were washed 3 times with tissue culture medium and finally refed with the medium, the addition of human gamma-globulin with a 1:100 herpes virus-neutralizing titer had no protective effect on the amount of syncytium formation produced after incubation for another 5 hours at 37°C.

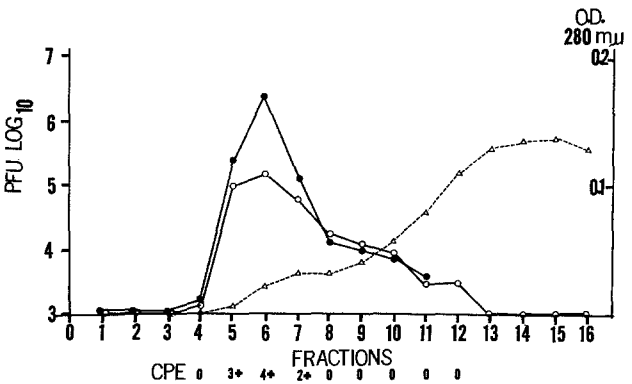

Fig. 2. Biogel filtration of herpesvirus. Fractions 1–3 contained the void volume. The results of 2 repeated experiments are shown in (●———●) and (○———○) in plaque forming units. Optical density is shown in (△-----△).

Gel Filtration

The syncytium-producing components were isolated in a purified form with Sephadex G-200 gel. Thus after concentration of the crude virus by ultrafiltration and passage through Sephadex gel 50% of the original syncytium-producing activity along with only 1/70 of the original protein content was obtained in the void volume. The results of gel filtration through Biogel A-50m are shown in Fig. 2, in which it can be seen that syncytium-producing components consisted largely of particles of the same size as the infectious virus particles but also included some slightly smaller particles. After simple filtration through a Nalgene membrane 75% of the syncytium-producing components was recovered.

Density Gradient Centrifugation

When 4-fold concentration of the crude virus was analysed for density in sucrose, it was found that the syncytium-producing activity was

present in materials with a wide range of densities (Fig. 3), including a component with the same density as the infectious virus (1.27 g/ml), a component with a slightly lower density (1.20 g/ml) and a low-density component (1.10 g/ml) with a density closely corresponding to that of soluble complement-fixing antigens of this virus (17). The low-density components (1.10—1.15 g/ml) appeared to have the same density range as that of the serum lipoproteins (20). Such density gradient centrifugation pattern was quite similar to that of the smaller measles virus hemolysins (9), which also produce a syncytium.

When the virus was concentrated by ordinary centrifugation (16) and resuspended in 1/25 of the original volume of crude virus preparation

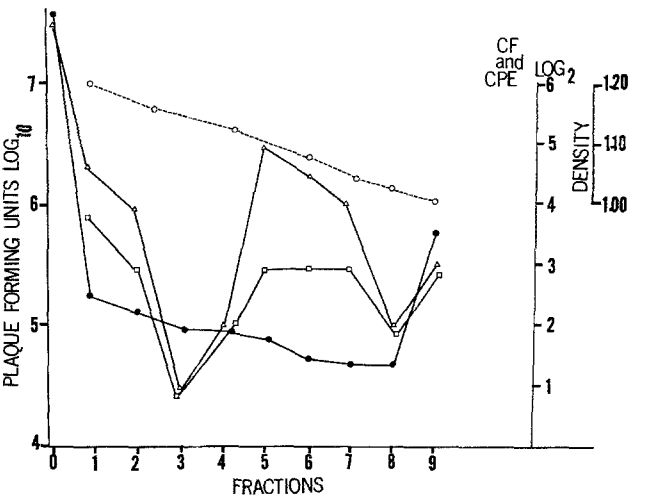

Fig. 3. Density gradient centrifugation of herpesvirus in sucrose. Density (○ ····· ○), infectivity in p.f.u. (●——●), complement fixing activity (CF) (△——△) and cytopathic effect (□——□).

having 16 syncytium-producing units/ml, there resulted a 16-fold concentration of infectivity but only a 4-fold increase in the syncytium-producing activity. This may indicate that the syncytium-producing activity was either only partly sedimented with the virus or that it was more readily inactivated by centrifugation.

Effects of Temperature and Storage

The syncytium-producing activity was completely destroyed by heating at 56°C or higher for 20 minutes. The instability at various temperatures and under various storage conditions is shown in Table 3. The syncytium-producing component associated with infected cells was found to be more stable on storage than the extracellular syncytium-producing component contained in the culture fluid.

Effects of Freezing and Thawing

Although in general repeated freezing and thawing resulted in a loss of both infectivity and syncytium-producing activity (Fig. 4), in the particular case of the rapid freezing and thawing of small amounts of material there was in one experiment an actual increase in the syncytium-producing activity with a concurrent destruction of more than 99% of the infectivity; in another experiment, however, there was a loss of both activities. When crude virus in infected tissue culture fluid (16)

Table 3. Instability of Syncytium-producing Components at Various Temperature

Time in hours	Infectivity p.f.u. at 37° C %	Temperature		
		37° C	26° C	4° C
		Syncytium-producing units		
0	100	32	32	32
2	40	32	32	32
3	20	32	32	32
4	15	16	32	32
9	2	4	4	4
18	0.03	2	2	2
24	0.01	1	1	1
48	0.01	0	0	0

Table 4. Irradiation of Syncytium-producing Components by Ultraviolet Light

Irradiation time in seconds	Virus infectivity in p.f.u. %	Syncytium-producing units titrated in	
		Tubes without added active virus	Tubes with added active virus
0	100	32	32
30	1.2	4	4
60	0.04	2	2
90	0.02	0	0

which contained 5% of calf serum as a nutrient was frozen and stored at −10°C, −20°C and −70°C, the recovery after 1 week was 5% or less in syncytium-producing units. When infected cells were stored under these conditions and in the same medium the recovery was 5—20% of the original material.

Effects of Irradiation with Ultraviolet Light

Table 4 shows the rates of inactivation of the infectivity and of the syncytium-producing activity of herpes simplex virus by ultraviolet light. Here again the syncytium-producing activity was slightly more

resistant than the infectivity (15). Partially ultraviolet-inactivated virus was then titrated for syncytium-producing activity in cell cultures with and without the simultaneous addition of an amount of fully active virus equivalent to 0.1 syncytium-producing unit. If the active infection of the cells contributed to the formation of syncytia, those cultures with the additional active virus would have shown a higher titer of syncytium-producing activity. Since no such difference in the titer was found, it can be concluded that an infection with the above-mentioned amount of active virus played no role in the formation of syncytia under these conditions.

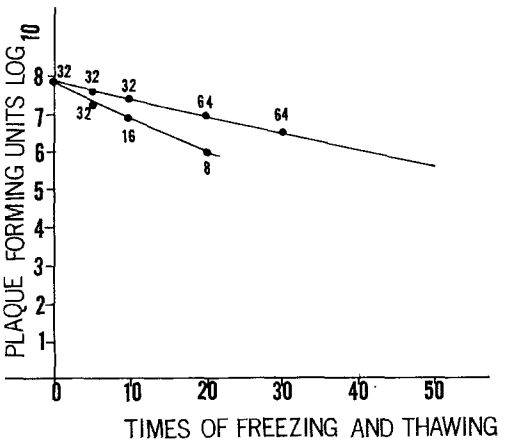

Fig. 4. Freezing and thawing of herpesvirus. The results of two experiments. Infectivity (●——●). The numbers along infectivity titrations indicate the syncytium producing units of each preparation.

Effect of pH

The syncytium-producing activity was stable over a wide range of pH of 5 to 9, but beyond these limits the activity was destroyed very rapidly. A slightly more rapid loss of infectivity also occurred at pH 4.3

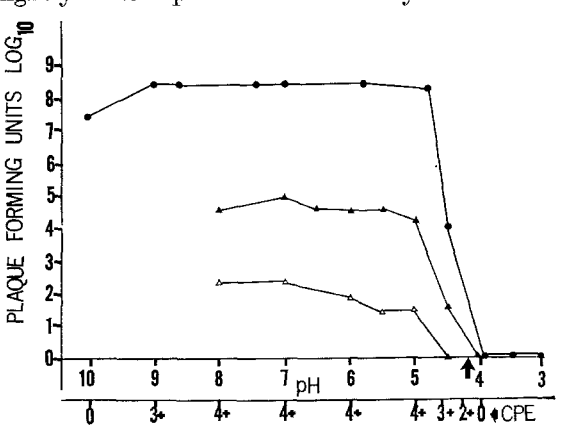

Fig. 5. Effect of pH on herpesvirus. All 3 curves indicate infectivity titrations obtained in 3 different preparations. The arrow shows the point of the complete loss of infectivity.

to 4.5 (Fig. 5). When the original infectious virus titer was less than 100 p.f.u./ml complete inactivation of infectivity was produced at pH 4.5. At pH 4.3 the virus infectivity of all preparations was completely

destroyed and only a small amount of syncytium-producing activity remained. However, even below this pH a number of the soluble herpes simplex virus antigens are stable. Thus the precipitin patterns shown in Fig. 6 indicate that 4 of 7 original precipitins were stable at pH 2; 3 of the others were destroyed at pH values of 5, 4 and 3, respectively (not shown in Fig. 6). One of the latter 3 dilute acid-labile antigens was isolated by chromatographic procedures which will be reported elsewhere.

Reactivity with Chemical Agents

Protein-precipitating agents such as 50% ammonium sulfate and sodium sulfate were found to precipitate the syncytium-producing components with a recovery of 33% of the original material. Lipid solvents

Table 5. Effects of Various Enzymes and Biologic Preparations on Syncytium-producing Components of Herpesvirus

Enzymes and biologic preparations	Infectivity of virus p.f.u./ml at concentration of 240 µg/ml	Syncytium-producing units at the concentration of materials in µg/ml			
		240	64	15	4
Untreated control	$10^{8.5}$	32	32	32	32
Trypsin	$10^{5.4}$	16	32	32	32
Chymotrypsin	$10^{5.8}$	0	0	16	32
Ficin	$10^{6.5}$	8	8	16	32
Alkaline phosphatase	$10^{8.0}$	32	32	32	32
Acid phosphatase	$10^{6.5}$	8	32	32	32
Phospholipase C	$10^{5.0}$	0	0	16	32
Phospholipase D	$10^{5.6}$	8	8	8	32
Lipase	$10^{6.8}$	8	8	16	32
Ribonuclease	$10^{8.5}$	32	32	32	32
Deoxyribonuclease	$10^{8.3}$	16	32	32	32
Heparin	$<10^{2.0}$	0	0	8	16
Tetracycline	$10^{8.5}$	32	32	32	32

such as methanol, chloroform and acetone were found to destroy the syncytium-producing activity. Shaking with ether for only 30 seconds resulted in a complete loss of syncytium-producing components as well as of infectivity. Treatment with Freon for 30 seconds resulted in a 25% recovery. The procedures used for treatment with the above agents were described in a previous article dealing with the soluble antigens of herpesvirus (17).

Effects of Enzymes and Other Biological Preparations

The syncytium-producing activity was highly susceptible to phospholipases, whereas it was varyingly susceptible to proteolytic enzymes, which suggests that it is dependent for its activity on the integrity of a lipoprotein structure. Although the infectivity of the herpesvirus is

quite susceptible to trypsin and phosphatases, these enzymes had little effect on the syncytium-producing activity (21—22). Herpesvirus infectivity is inhibited by heparin (23). Heparin also inhibited the syncytium-producing activity. It is known that heparin inhibits the activities of lipoproteins by various mechanisms (24). Tetracycline, which neutralizes rickettsial toxins in vitro (2), had no effect on the syncytium-producing components. Like the bacterial endotoxins, the syncytium-producing activity of the herpes simplex virus was inactivated by fresh guinea pig serum when it was not diluted more than 1:2.

Preincubation of crude virus with phospholipid preparation (Lecithin RG) did not decrease syncytium-producing activity even when as much as 3 mg/ml were used. When rabbit kidney tissue culture cell tubes

Table 6. Difference in Susceptibility of Various Cell Line Cultures to Syncytium-producing Component of Herpesvirus[1]

Types of tissue culture cells	Types of cytopathic effect	Time of 50% cytopathic effects
Primary rabbit kidney	extensive syncytia[2]	3 hours 20 minutes
Human embryo kidney	rounding	1 hour 40 minutes
Human diploid	rounding	4 hours 40 minutes
Human amnion	proliferative and rounding	3 hours 40 minutes
Baby hamster kidney	small syncytia and rounding	3 hours 40 minutes
Mouse MCN	rounding	5 hours 30 minutes

[1] 32 Units were added to all culture tubes.

[2] Pictures of cells for Fig. 7 were taken from tubes fixed and stained approximately 1~2 hours after the time shown here.

were inoculated with higher than 50 μg/ml concentration of lysolecithin and incubated at 37°C for an hour, there was a degenerative rounding of cells due to the lysolecithin (General Biochemicals, Chagrin Falls, Ohio).

Activation of Lysosomal Enzymes

The latter enzymes are activated when cells are injured by various viruses (25—26). The syncytial giant cells here produced by crude herpesvirus in the kidney cells were found to stain strongly for acid phosphatase in the region of the central cluster of nuclei.

Effects of Cations

When either $MgCl_2$ or $CaCl_2$ was added to tube cultures to a final concentration of 0.02 *M*, the formation of syncytium was quite markedly inhibited, whereas it was not inhibited below 0.008 *M*. Thermal inactivation (27) of crude herpesvirus at 37°C for 60 minutes resulted in decrease of infectivity from $10^{7.5}$ to $10^{5.3}$ p.f.u. with a concomitant loss

of syncytium-producing activity at 1*M* concentration of Mg^{++}, but both activities were intact below 0.1 *M*. When crude virus was added to tube cultures and adsorbed for 60 minutes at 37°C in the presence of 0.01 *M* amounts of Mg^{++}, the adsorption efficiency was not different from controls, as evidenced by the equality of the time of formation of syncytium after the medium was replaced after 60 minutes adsorption period. However, syncytium formation was significantly inhibited when the medium in the tubes was replaced at above time with medium containing 0.01 *M* Mg^{++}. Inhibitory effects of some cations on hemolysis by mumps virus have been previously described (10).

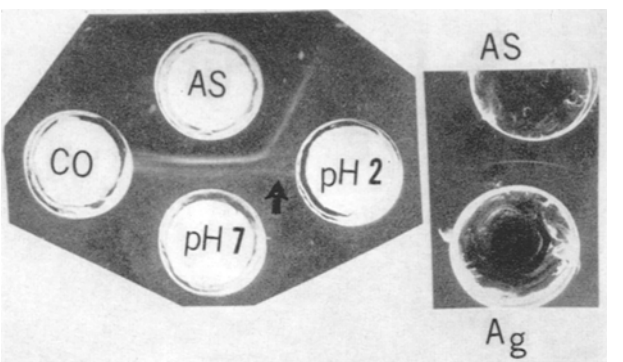

Fig. 6. Precipitating antigens of herpesvirus. The left-hand figure shows 7 precipitin bands formed between antiserum (AS) and antigens at pH 7 and 4 bands between antiserum and antigens treated at pH 2 in direct observation of the plates. The arrow indicates the bands which were lost at pH 2. In the right-hand figure one of the antigens marked by arrow in the left hand figure was purified by chromatographic procedure and shown for the comparison. Antigen = Ag. CO = control antigens (16).

Optimal Age of the Cells and Optimal Medium for Producing the Syncytia

The optimal age of the cells was 5—7 days, especially when they were given a feeding of fresh culture medium, and the pH of the culture medium was 7.0—7.4. Cells more than 8 days old tended to show a degenerative rounding. Addition of 3% glucose at the time of the inoculation of crude virus did not increase the titer of syncytium-producing activity and the use of 25% or more calf serum in the medium had a slight inhibitory effect on the formation of syncytia. When nitrogen gas was passed through the culture medium and it was then covered with mineral oil to create anaerobic conditions, there was a greater amount of degenerative rounding of the cells.

Susceptibility of Various Cell Lines to the Cytopathic Effects

Table 6 shows the differences in the time of the first appearance of the cytopathic effects and the types of cellular changes such as degenerative proliferation and rounding in different cell lines (Fig. 7.) Human

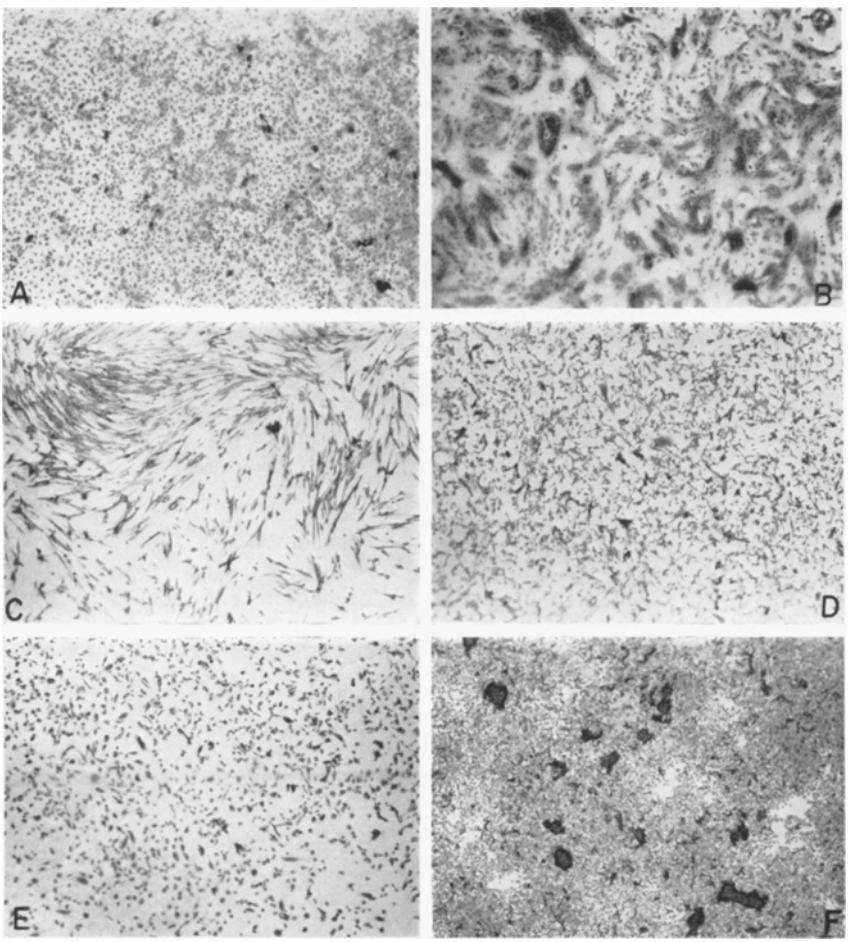

Fig. 7. Cytopathic effects of herpesvirus in various tissue culture cells. For explanation see text.

A = uninfected rabbit kidney cells. B = syncytium formation in rabbit kidney cells.
C = infected human diploid cells. D = infected baby hamster kidney cells.
E = infected human embryo kidney cells. F = infected human amnion cells.

All tube culture cells stained by Giemsa stain. Magnification 20 ×.

embryo kidney cells did not exhibit syncytial changes (syncytium-producing components, 32 units) but were highly susceptible to the cytopathic effect of herpes simplex virus. When 8 units of syncytium-producing components were added to the culture of human embryo kidney cells instead of 32 units, the first appearance of the cytopathic effect in the form of degenerative rounding of the cells was delayed by 4 hours. Human amnion cells responded to the active components with a striking cellular proliferation as previously seen in other cells when they were infected by herpes simplex virus (12, 28).

Susceptibility of Leukocytes to Lethal Effect of the Syncytium-producing Activity

The peritoneal exudates of mice and guinea pigs were found to contain leukocytes which were killed by the syncytium-producing activity and those which were not as measured by the uptake of Evans blue dye (Fig. 8).

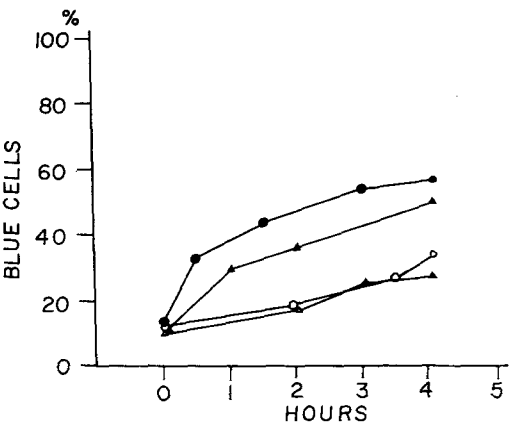

Fig. 8. Lethal effect of syncytium-producing components on leukocytes. Peritoneal exudate leukocytes from guinea pigs with (●———●) and without (○———○) addition of components. Those from mice are shown in (▲———▲) and (△———△), respectively. Blue cells indicate dead cells stained by Evans blue.

Discussion

The syncytium-producing components of herpes simplex virus appear to be among the most labile moieties of this virus and are probably lipoprotein in nature. When the virus particle is disintegrated into smaller units this component may be even less stable than when it is in intact infectious virus. However, since the activities of biological materials are often greater when these materials are in a soluble form than when they are in a particulate form it may be that the syncytium-producing components are more active in a soluble form when they are released from the virus particle close to their target in the mammalian cells or from the infected cells which produced such components in excess.

Herpes simplex virus is known to contain relatively large amounts of phospholipids (22%) along with 70% proteins, 1.6% carbohydrates and 6.5% deoxyribonucleic acid (29). The phospholipids of the influenza virus differ from those of the host cells in possessing a high content of phosphatidic acid in their phospholipid structure. Interestingly enough, the more strongly cytopathic strains of this virus have a higher content

of this acid than ordinary strains when grown in calf kidney tissue culture cells (30).

That the toxicity of the herpes simplex virus is comparable to that of various bacterial toxins is shown by the fact that in rabbit kidney cell cultures one syncytium-producing unit per ml of herpesvirus material was equal to 0.003 μg/ml of diphtheria toxin, 30 guinea pig minimum lethal doses/ml of tetanus toxin and 3 minimum lethal doses of staphylococcal alpha-toxin (31). None of the above mentioned bacterial toxins produced syncytia.

The formation of syncytial giant cells has been one of the most frequent histopathological findings in human lesions produced by the herpes simplex virus and the specificity of this finding has been used for diagnostic purposes. The specificity of the syncytium-producing effect of the herpesvirus, in contrast with its effect in producing degenerative rounding of cells, which may also be produced by nonspecific materials, may prove of value for quantitative determination of the specific activity of toxic components of this virus.

There are instances in which giant cells and rounded cells obtained from late lesions of herpetic human skin and mucous membranes do not stain strongly with fluorescent antibody, whereas those from the earlier lesions stain. It would seem probable from the foregoing data that such histopathological changes could have been induced by secondary action of herpesvirus toxins which were originally produced at the site of the earlier lesion.

Summary

The syncytium-producing activity of herpes simplex virus was shown to be a result of a toxic activity of certain viral components present on the viral particles as well as free. The physicochemical properties of the syncytium-producing components of herpesvirus suggest that they are lipoprotein in nature. The rapid appearance of the syncytium-producing activity in tissue culture prior to the appearance of infectious virus (32, 33) and the presence of antibodies in human and rabbit sera was demonstrated. The lability of syncytium-producing activity to pH values below 4 or above 10, heating at 56°C for 20 minutes, UV irradiation, repeated freezing and thawing and various chemical agents such as ether and acetone, to all of which certain other antigenic components of this virus are stable, was demonstrated. Syncytia appeared best in primary rabbit kidney, less well in baby hamster kidney and not in human embryo kidney, diploid or amnion cells or mouse MCN cells.

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