THE RELATION OF HERPES VIRUS TO THE CELL NUCLEUS*

THOMAS FRANCIS, JR. AND HILDA B. KURTZ

The selectivity with which viruses tend to attack specific tissues and cells, resulting in characteristic types of morphologic alteration, readily invites investigation of the manner in which the virus becomes attached to the cell. Infection with the virus of herpes simplex is typified by the presence of inclusion bodies in the nucleus which have been thought by some investigators to represent the localized virus. This possibility is well discussed in van Rooyen and Rhodes.[17]

In recent years increasing use has been made of centrifugation as a method for separating the structural units of cells.[5] It was thought, then, that similar procedures could be employed to determine whether herpes virus has a selective affinity for the nuclei of susceptible cells and to ascertain whether the virus is intimately related to the nucleoprotein of the nucleus.

The present paper is in reality a progress report including a description of the experimental approach and an interpretation of the data which have been obtained.

Materials and methods

1. *Preparation of virus.* Two different strains of herpes simplex virus were used. The J. R. Smith strain was maintained through seven passages in mouse brain after being received in our laboratory from Dr. J. E. Smadel of the Army Medical Department Research and Graduate School, Army Medical Center. Eight mouse brains, removed three days after infection, were ground and made into a 10 per cent suspension in 10 per cent horse serum-saline. After centrifugation at 1500 rpm for 20 minutes, 3-4 cc. were placed in small ampules which were sealed with heat and frozen in a dry ice cabinet. Culture of the suspension was negative for bacteria after 48 hours. Titration, done shortly after freezing, by intracerebral inocultion of mice showed a 50 per cent end point[14] of $10^{-4.6}$. Based upon this evaluation approximately 1000 LD50's were employed in the tests. It can be pointed out, however, that the virus is not uniformly stable under these conditions of storage and variation in the actual amount of virus resulted.

* From the Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Michigan. Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

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The Armstrong strain of virus was received from Dr. Charles Armstrong of the National Institutes of Health as mouse brain from the forty-eighth passage. It was adapted by repeated passage on the chorio-allantoic membrane of the embryonate egg until it produced gross lesions in the liver and heart of the embryonic chick.[2] Pools of virus were prepared from infected membranes of the twenty-fifth or later passage collected on the third or fourth day after inoculation of 10-12-day embryos. The membranes were ground, and 10 per cent suspensions were made in 10 per cent horse serum-saline. After centrifugation, the supernatant was sealed in glass ampules and frozen. Tests for sterility were made, and the titer of virus then determined by intracerebral inoculation of mice. The titers attained by the twenty-fifth and twenty-eighth passage material were, respectively, $10^{-4.88}$ and $10^{-4.75}$. Sometime after this the virus appeared to be losing its virulence for mice and consequently was passed through the brains of mice for five passages and then transferred back to eggs. Pools from the second and the sixth subsequent passages in eggs had titers in mice of $10^{-5.5}$.

The titrations were done by intracerebral inoculation of groups of six mice which were observed for 21 days and the fatalities recorded. The LD50 was calculated upon deaths in the first 14 days, since after that time only occasional irregular deaths were observed.

2. *Preparation of cell fractions from embryonic chick liver:* (a) *In hypotonic salt solution.* This procedure for obtaining nuclei is similar to that described by Hoerr[9] as a modification of Dounce's procedure.[8] All containers, apparatus, and reagents were chilled, and all operations were so far as possible carried out in a cold room. All centrifugations were performed in the cold (—6°C).

Livers of 14-15-day embryos were collected aseptically in a petri dish after allowing the embryos to bleed freely. The livers were chilled immediately in an ice bath. They were then rinsed in isotonic salt solution to wash off adhering blood, drained, and minced finely with curved scissors. Working in a refrigerated room, the minced livers were ground lightly in small amounts in small mortars, adding gradually 0.6 per cent NaCl solution buffered to pH 6.1 with 0.01M PO_4. A final concentration of 1.0 gm. of liver to 5.0 cc. of solution was prepared, the suspension was strained through four layers of gauze, and the fluid portion was centrifuged for 20 minutes at 2000 rpm in a horizontal centrifuge. The supernatant fluid was carefully decanted and the sediment, containing nuclei, red cells, liver cells, and debris, was resuspended in the original volume of hypotonic saline, taking care to stir gently so as not to rupture the nuclear membranes. The material was centrifuged for 15 minutes at 1500 rpm and the supernatant poured off. The residue was again resuspended in the original volume of saline and centrifuged for 10 minutes at 1500 rpm. The sedimented nuclei were then resuspended in one half the volume and centrifuged 5 minutes at 1000 rpm. The supernatant was discarded, the residue resuspended again in one half volume and centrifuged for 3 minutes at 800 rpm; this was done three times. The nuclei were then suspended in the original volume of hypotonic saline and allowed to settle in a tall cylinder for 45 minutes in the cold. The supernatant, containing the nuclei, was carefully removed by pipette and the nuclei recovered by centrifugation for 3 minutes at 800 rpm. The volume of packed sediment was noted and a 10 per cent suspension was prepared in 10 per cent horse serum-saline. The number of nuclei in a preparation was then determined by the addition of methyl green and counting in a hemacytometer. On the average, approximately 31,000 nuclei were counted per cu. mm. A suspension

for comparison was prepared by grinding whole embryonic livers thoroughly in mortars so as to break cellular structures and to free the component materials. A 20 per cent suspension was made in 0.6 per cent NaCl solution pH 6.1. The suspension was centrifuged at 1000 rpm for 1 minute, the supernatant collected and recentrifuged at 3000 rpm for 5 minutes. The sediment was resuspended in 10 per cent horse serum-saline to form a 10 per cent suspension and used in this form.

(b) *In sucrose.* This procedure which was used primarily for fractionation of infected livers is an extension of that described by Hogeboom, Schneider, and Pallade[10] for the preparaiton of mitochondria. It was carried out with sterile technic at 4°C. After bleeding, the livers are removed from 15-day chick embryos 4 days after infection with Armstrong strain virus and placed in cold 0.85 per cent NaCl. They are then drained on gauze and weighed. They are minced with scissors, a 20 per cent suspension prepared in 30 per cent (0.88M) sucrose, and homogenized in a glass homogenizer[13] for 2 minutes at approximately 1000 rpm. The material is strained through four layers of gauze and the gauze washed with sufficient sucrose to regain the original volume. One half cc. volumes of sucrose are placed in graduated centrifuge tubes and the homogenate is carefully introduced so as to prevent mixing. The tubes are centrifuged at 1800 rpm for 20 minutes in a horizontal centrifuge. The supernatant is then carefully removed and serves as the source of mitochondria and microsomes. The sediment contains the hepatic nuclei, some whole hepatic cells, erythrocytes, and debris; its volume is noted in the graduated tube.

After straining through gauze an aliquot of the homogenate was removed and ground with alundum, suspended to 10 per cent in 30 per cent sucrose and centrifuged at 1800 rpm for 10 minutes. The supernatant was titrated and served as the virus control.

Mitochondria and microsomes. To remove any remaining nuclei the supernatant is centrifuged at 1800 rpm for 10 minutes three more times, the fluid being poured into a fresh tube each time. The final supernatant is centrifuged at 16,000 rpm for 20 minutes in an anglehead centrifuge and the fluid portion poured off and saved. This is referred to as the 20 per cent suspension of microsomes. No attempt to purify the microsomes is made since the speed of centrifugation required would also sediment the virus particles. The sediment is resuspended to the original volume in sucrose and recentrifuged at 16,000 rpm for 20 minutes. The supernatant is discarded and the sediment resuspended to the orignal volume as the 20 per cent suspension of mitochondria.

Nuclei. The sediment containing the nuclei is resuspended in 30 per cent sucrose, avoiding the red blood cells which have packed first at the bottom of the tube, and the suspension centrifuged at 1800 rpm for 15 minutes. This is done four times, the sediment being resuspended in fresh sucrose solution each time. The last centrifugation is done in graduated centrifuge tubes, and the amount of sediment noted. The 20 per cent suspension of nuclei is made by suspending the last sediment in a volume of sucrose corrected for amount of sediment lost during the process.

3. *Nucleoprotein extraction.* Desoxyribosenucleoprotein (DNP) was extracted from the nuclei of liver cells following the procedure of Mirsky and Pollister.[12] Sterile technic was employed and all operations were carried out at 4°C. Livers from 15-day embryos infected with herpes virus are transferred to ice-cold 0.85 per cent NaCl, then drained on gauze and weighed. They are minced with scissors, suspended to 30 per

cent in 0.85 per cent NaCl, and homogenized for one minute in a glass homogenizer at 1000 rpm. The homogenate is centrifuged for 10 minutes at 8000 rpm in an angle-head centrifuge in the cold, and the supernatant is poured off. The tissue residue is well mixed and suspended to the original volume in 0.85 per cent NaCl and centrifuged at the same speed. This process is repeated three times and presumably removes the great bulk of cytoplasmic material including the ribonucleoprotein (RNP). The sediment of washed tissue is suspended at 40 per cent concentration, in terms of the original weight, in 0.14M NaCl and an equal volume of 2M NaCl added to give a final concentration of 1M NaCl. The mixture, which is quite viscous, is stirred rapidly over night. After 18 to 20 hours, it is centrifuged at 10,000 rpm for one hour. The viscous supernatant fluid containing the DNP is removed, its volume measured, and the nucleoprotein precipitated by pouring it into six volumes of distilled water. The precipitate is collected on a glass rod and transferred to a graduated centrifuge tube so as to measure the volume of the precipitate and the accompanying fluid. To redissolve the nucleoprotein an equal volume of 2M NaCl is added and then sufficient 1M NaCl to bring back to the volume of the extract. It is placed on the stirrer to facilitate solution. When all the material is dissolved, it is again centrifuged at 10,000 rpm for one hour and the supernatant saved. In two experiments the nucleoprotein was precipitated, dissolved, and centrifuged a second time.

The *quantitative determination* of the DNP was made as desoxyribosenucleic acid (DNA) employing the method of Stumpf[16] and the extraction procedure of Schneider[15]. In one instance, for comparison, the method of Dische[7] was used.

Experimental

The first experiments were devised to determine whether herpes virus is selectively adsorbed to nuclei of apparently susceptible cells. A volume of 4.0 cc. of a suspension of nuclei containing, on the average, approximately 31,000,000 nuclei per cc. was mixed with an equal volume of virus suspension so as to yield a final virus titer of 500-1000 LD50 per unit volume. A similar preparation of virus and the resuspended sediment of ground whole liver was made. A control of the same final concentration of virus alone in 10 per cent horse serum-saline was retained. The materials were kept at 4°C. Immediately after mixing, again 4 hours and 24 hours later, samples of the individual suspensions were removed and centrifuged at the same speeds employed in their final preparation so as to sediment the particles. The supernatant fluids were then titrated intracerebrally with fourfold dilutions to determine whether virus was removed from the fluid presumably by attachment to the available cellular material. If virus were selectively adsorbed to nuclei, it should be present in reduced amounts in the remaining supernatant fluid.

The average results of three such experiments are presented in Table 1. Two were done with J. R. Smith strain from mouse brain and one with

the Armstrong strain from chick embryo. Although there was an absence of numerical uniformity in the titers obtained, owing largely to the narrow range of virus concentration concerned, two consistent trends were noted.

TABLE 1

TESTS FOR ADSORPTION OF HERPES VIRUS BY NUCLEI

Time of sample (hrs.)	*Titer of supernatants at 4°C.**		
	Virus alone	*Virus + nuclei*	*Virus + ground liver*
0	470	335	140
4	330	245	100
24	360	270	75

* Average of 3 experiments.

The first was that the nuclei did not unite with virus to remove it in significant amounts from the suspending fluid. Secondly, the preparation of ground liver consistently reduced the amount of virus remaining in the fluid.

Although the evidence presented clearly indicates that herpes virus does not selectively combine with the nuclei separated from normal cells, the possibility remains that the virus may enter the nucleus of the whole cell by way of the cytoplasm. Efforts to demonstrate the distribution of virus in relation to different structural components were, therefore, made with nuclei, mitochondria, and microsomes of infected tissue prepared in sucrose by centrifugation (Table 2).

TABLE 2

RELATION OF VIRUS TO CELLULAR STRUCTURES OF INFECTED CELLS

	Experiment no.	
	14	*15*
Ground liver	56,000	147,000
Nuclei	32,000	25,000
Mitochondria	43,000	146,000
Microsomes	56,000	251,000

The results of two experiments are presented in Table 2. In Experiment 14 no significant difference was observed in the virus accompaniment of the different structures although that of the nuclei was the lowest. In the next

experiment, however, the nuclear preparation had a distinctly lower content of virus than the other fractions, again demonstrating that the virus was not selectively related to the cell nuclei. Interestingly, the preparations of microsomes which presumably contain the major portion of RNA[4] had the highest titers of virus.

A third type of experiment sought to determine the relation of virus activity to the DNP content of infected embryonic liver. Studies to date have shown that some viruses of animal origin contain primarily DNA.[3,11]

TABLE 3

RELATION BETWEEN DNP CONTENT AND VIRAL ACTIVITY OF EMBRYONIC LIVER INFECTED WITH HERPES VIRUS

	Experiment 25		*Experiment 26*	
		DNA		
Material	*Virus*	*γ/cc./10%*	*Virus*	*DNA*
Homogenized infected liver				
Washed with 0.14M NaCl				
First wash	562,000	23		
Fourth wash	3,200	0		
Washed tissue	40,000	250	100,000	256
Ground and centrifuged (control)				
Washed tissue				
In M/1 NaCl 4°C., 20 hours				
↓ Total mixture	316,000	305	251,000	325
Centrifugation				
↓				
Crude DNP—Supernatant	100,000	283	17,800	218
Sediment	178,000	60	43,000	31
↓				
Ppt. in 0.14M NaCl				
Redissolve in M/1 NaCl				
Centrifuged				
DNP—Supernatant	13,300	265	10,000	193
Sediment	4,000	0	4,900	7
Virus control				
Whole liver ground in M/1 NaCl				
Centrifuged 8,000 rpm — supernatant, 4°C.				
0 hours	143,000	195		
24 hours	251,000			

Furthermore, the DNP or DNA of the animal cell appears to be essentially nuclear in location. Because the nuclei constitute the major source of the cell's DNP and because herpes virus is an animal virus which might be expected to be DNA in type and which produces inclusions in the nuclei, it was thought that extraction of the DNP of infected tissue would furnish information as to whether the two are intimately related.

The livers from infected chick embryos were homogenized, washed free of cytoplasmic material, and extraction of nucleoprotein was done in molar NaCl solution. Preliminary tests demonstrated that the virus remained active in solutions of this strength. Titrations of virus in mice, and DNA determinations were carried out at different stages upon the fluid and sedimented parts of the preparations. The supernatant fluids were removed from suspensions centrifuged at 10,000 rpm for one hour. The sediments were then ground and the supernatant tested after centrifugation at 8000 rpm for ten minutes. Six experiments of this nature were done with virus concentrations varying from LD50's of 10^{-4} to 10^{-6} and the results of two typifying the procedure are presented in Table 3.

TABLE 4

ADDITIONAL DETERMINATIONS OF DNP CONTENT AND VIRAL ACTIVITY

	M/1 NaCl extract				*DNP 1x ppt. + diss.*	
	Crude DNP		*Sediment*			
Experiment	*Virus*	*DNA*	*Virus*	*DNA*	*Virus*	*DNA*
12	>2500	222	4000	72	100	156
13	5600	192	4000	34	750	182
16	25,300	261	20,100	18	2700	231
23	39,000	199	*1350	13	2512	177

* Centrifuged at 10,000 rpm 1 hour instead of 8000 rpm 10 minutes.

The data herein presented show a definite dissociation between the DNP content of the tissue or its extracts and the amount of virus detectable in them. The first washing constantly had a high titer of virus and a low DNA content. The extracted nucleoprotein showed a reduced titer of virus although the DNA determinations were consistently high; moreover, the virus titer of the tissue from which the DNP had been largely extracted was greater than that of the fluid DNP.

From four earlier experiments the data in Table 4 were obtained. They emphasize the fact that there is a disproportion between the DNA content

of material extracted from infected tissue, presumably as DNP, and the viral activity; that the DNA remained largely in the soluble extract, whereas the sediment possessed about the same amount of infectious virus with a much lower content of DNA.

Discussion

These investigations were undertaken in the expectation that evidence could be gained as to the localization of herpes virus in the cell which it infects. The development of nuclear inclusions in the infected tissues has resulted in a widespread opinion that the inclusion represents a selective localization of the virus. And it was this concept which suggested the present studies. Contrary to expectations, however, the evidence obtained indicates that herpes virus is not primarily associated with the cell's nucleus. Under the conditions of experiment the data showed:

1. That herpes virus did not selectively combine with washed nuclei of apparently susceptible cells;
2. That herpes virus was not found in greater concentration in the nuclei of infected cells than in preparations containing primarily mitochondria or microsomes;
3. That the nucleoprotein extracted from the nuclei of infected cells is not proportionately related to the amount of active virus demonstrable. In fact, purification of the nucleoprotein by simply precipitating and redissolving it results in a great diminution in virus titer while the yield of nucleoprotein is changed but little.

It has been repeatedly noted that herpes virus is readily released from infected cells. The observations of the present study are in keeping with that experience since it was noted that washing of infected tissue which removes the great bulk of cytoplasmic material, including RNP, also takes out a high proportion of active virus. Moreover, since this washing does not seriously affect the nuclear content of DNP, the results suggest that the virus is more richly associated with cytoplasmic material.

Certain obvious questions concerning interpretations can be raised. First, is the failure of the separated nuclei to combine with virus merely a reflection of altered physiological state incident to the process of preparation? In this respect one can state that they appear relatively unchanged in staining characteristics. Furthermore, most work in which specific cell-virus union has been demonstrated *in vitro* has been done with material which has been subjected to washing and which is not in its normal physiologic environment. A second question relates to the possibility that the infected nuclei are broken up so that the material most likely associated with virus

is washed away. The nuclei remaining or the nucleoprotein extracted would represent, in this case, the unaffected cells of the tissue. The amount of DNP obtained, however, from the infected embryonic liver is essentially the same as that derived by the same procedures from normal liver, and the number of nuclei obtained from normal and infected liver is approximately the same.[1] Furthermore, sections reveal a high proportion of affected cells so that in some areas nearly all the nuclei contain inclusions. A third question is whether the quantity of DNP which might be virus is so small that it would of necessity be but a small fraction of the extracted nucleoprotein. This is expected to be the case but even so, some parallel between the total amount of DNP and the virus titer should be expected. And if the virus were composed primarily of DNP of nuclear origin, the two should accompany one another quantitatively. The results, however, lead to the conclusion that the virus activity is not related to the DNP but to material of other nature. This is in accord with the interpretation of Cowdry who pointed out that the inclusions of herpes did not show Feulgen reaction.[6] They indicate, too, that the nuclear inclusions are not virus itself but are changes perhaps secondary to virus injury at the cell surface or in the cytoplasm.

Conclusions

Although the inclusions produced by herpes virus are nuclear in location, no evidence was obtained to indicate that herpes virus is selectively bound to nuclei or to nuclear nucleoprotein. The results indicate that the inclusions are not herpes virus.

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