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Cellular Compartmentalization of Herpesvirus Antigens During Viral Replication

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HEp-2 cells infected with herpes simplex virus develop five distinct immunofluorescent elements. Three (small nuclear granules, large nuclear granules, and an amorphous mass filling the nucleus) contain antigens which react with a rabbit serum prepared against boiled infected cell debris. A labeled pool of human antibody revealed antigens making up cytoplasmic granules and those responsible for a diffuse cytoplasmic fluorescence. All five immunofluorescent elements are demonstrable with a rabbit serum prepared against unheated infected cell debris. Viral antigens are segregated in the nucleus or in the cytoplasm; within the limits of detection, each antigen accumulates in one compartment only. The antigens responsible for the diffuse cytoplasmic fluorescence and for the amorphous nuclear mass are synthesized early in infection; they are formed in arginine-deprived cells and exist in a form which does not sediment on centrifugation at 79,000 × *g* for 2 hr. The antigens comprising the nuclear and cytoplasmic granules arise relatively late in infection; they are not formed in arginine-deprived cells, and they are readily sedimented on centrifugation at 79,000 × *g* for 2 hr. Heating (60 C for 2 hr) confers on one or more cytoplasmic viral antigens a new specificity; the altered antigens are demonstrable with labeled rabbit anti-boiled infected cell serum which normally does not combine with cytoplasmic antigens.

The studies described in this paper deal with two aspects of productive infection of mammalian cells with herpes simplex virus (HSV). The first concerns the physical properties and morphological manifestations of HSV antigens in infected cells. The data show that viral antigens form at least five immunofluorescent elements differing from each other with respect to physical properties and immunological specificity. The second aspect of this paper deals with the intracellular localization of HSV antigens. The data indicate that viral antigens are segregated in the nucleus or in the cytoplasm and that, within the limits of detection, each antigen accumulates in one compartment only.

The findings reported in this paper are based entirely on the use of two antisera whose range of antibodies against products specified by virus do not overlap in immunofluorescence tests. The first is a commercial pool of human γ-globulin capable of neutralizing virus to a high titer. The labeled human antibody reacts exclusively with antigens located in the cytoplasm of infected cells. The second antiserum was made in rabbits immunized with boiled infected cell debris. The labeled rabbit antibody reacts with antigens located in the nucleus of the infected cell. The circumstances leading to the production of this antiserum were reported elsewhere (7). Briefly, one immunological manifestation of infection is the appearance of a new antigen on the surface of infected cells (6). The antigen is readily demonstrable in cytolytic tests with anti-infected cell serum and complement (12). The nature of the surface antigen is uncertain; the rabbit serum against the boiled cell debris was made in an attempt to determine its properties.

MATERIALS AND METHODS

Solutions and media. The composition of the following solutions and media were given in a preceding paper (9): PBS, phosphate-buffered saline; PBS-A, PBS containing 0.2% bovine albumin; PBS-A-γ-globulin, PBS-A containing 0.4% human pooled γ-globulin (Lederle Laboratories, Pearl River, N.Y.); 199-1, maintenance medium consisting of medium 199 and calf serum; 199-1-γ-globulin, liquid overlay used for assay of HSV in HEp-2 monolayer cultures. MEM, minimal essential medium of Eagle (1), with and without arginine, was obtained from Grand Island Biological Co., Grand Island, N.Y.

Cells. Human epidermoid carcinoma no. 2 (HEp-2) cells were obtained initially from Microbiological

Associates, Inc., Bethesda, Md., and were propagated serially in MEM containing 10% calf serum.

Virus. The macroplaque (MP) strain of HSV causes the formation of polykaryocytes in infected HEp-2 cell cultures. It is scored in terms of polykaryocyte-forming units (PoFU). The virus is assayed under 199-1-γ-globulin overlay (11). The properties of this virus have been described (11, 13).

Preparation of antisera. The sources of antibody for labeling were (i) a pool of commercial human γ-globulin (Lederle) especially selected for this study and designated Hu-p, (ii) a rabbit hyperimmune serum designated Ra-BIC and made against boiled infected HEp-2 cell debris, and (iii) a rabbit immune serum made against infected HEp-2 cell debris. The pooled human γ-globulin was selected on the basis of the observation that it does not react in immunofluorescence tests with antigens located in the nucleus. In this respect the Hu-p antibody resembles the human immune serum used in an earlier study (8). Ra-BIC serum was prepared as follows. Approximately 6×10^8 HEp-2 cells that had been infected 72 hr previously were washed three times with 0.01 M PBS (*p*H 7.2), suspended in 100 ml of distilled water, and incubated for 1 hr at 40 C. At that time, the suspension was acidified with 1 ml of 1 N HCl and incubated for an additional 1 hr. The sediment obtained after the second extraction was washed three times with PBS, resuspended in 24 ml of buffer, and sealed in glass ampoules. These were weighted and submerged in boiling water for 90 min. After boiling, a 0.5-ml sample was tested for residual infectivity but none was found. The remainder was used for immunization of rabbits. The immunization schedule consisted of multiple intramuscular injections once a week for 6 weeks. The rabbits were bled 1 week after the last injection. The serum against infected HEp-2 cell debris was prepared in the same fashion except that the infected cell debris was not heated.

Conjugation. The globulin fraction recovered from rabbit sera with 6,9-diamino-2-ethoxyacridine lactate monohydrate (ethodin; Winthrop Laboratories, New York, N.Y.) and the human immune γ-globulin were conjugated to fluorescein isothiocyanate by the method of Riggs as described by Marshall et al. (3). Residual ethodin and fluorescein isothiocyanate were removed from the conjugated globulin by dialysis against isotonic saline buffered at *p*H 7.2 with 0.01 M phosphate and filtration through G-25 Sephadex. The labeled antisera were then absorbed repeatedly at 37 C with uninfected Hep-2 cell debris until undiluted as well as diluted conjugated preparations failed to stain uninfected HEp-2 cells.

Absorption of conjugated antibody with viral antigens. To determine the properties of viral antigens illuminated by immunofluorescence in situ, the Ra-BIC and Hu-p conjugated antibody were absorbed with fresh and boiled viral antigens fractionated according to sedimentation behavior. Details of the procedures for preparation of viral antigens are outlined in Fig. 1. Briefly, approximately 15 cm^3 of wet-packed cells, infected 48 hr previously, were suspended in 50 ml of PBS, frozen-thawed five times, and sonically disrupted; the debris (designated P1) was pelleted by

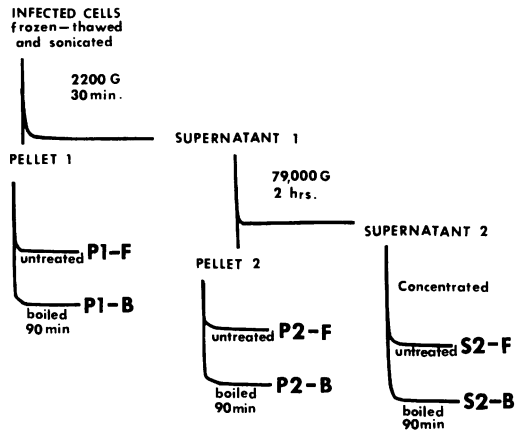

FIG. 1. *Procedure for preparation of viral antigen fractions used to absorb labeled antibody.*

low-speed centrifugation. The supernatant fluid was then centrifuged at high speed, yielding a supernatant fraction and a pellet designated S2 and P2, respectively. S2 was concentrated by freeze-drying, brought to 24 ml in volume with water, then made isotonic by dialysis against PBS. Pellets P1 and P2 were each resuspended in 25 ml of PBS and recentrifuged. At this point, one-half of P1, P2, and S2 fractions (designated with suffix -B) were boiled for 90 min. The untreated fractions were designated with the suffix -F. The fractions obtained then were P1-F, P1-B, S2-F, S2-B, P2-F, and P2-B.

The conjugated Ra-BIC and Hu-p antibody were absorbed as follows. A 1.5-ml amount of the appropriate virus antigen fraction was added to 2 ml of antibody in a tube (16 by 150 mm). The tube was then sealed and placed flat on a shaker at 37 C. Additional 1.5-ml amounts of the viral antigen fraction were added to the tube after 1, 2, 3 hr. After 4 hr of shaking the contents of each tube were centrifuged in a Spinco 40 rotor at $6{,}000 \times g$, for 30 min. The supernatant liquid containing absorbed antibody was collected and stored at −10 C. This procedure was repeated for each antigen fraction. As a control, one portion of each antibody preparation was diluted with PBS, then shaken and centrifuged in exactly the same fashion as the antibody absorbed with viral antigens.

Preparation and handling of cover-slip cultures. HEp-2 cells grown on no. 1.5 Corning cover slips (10 by 30 mm) in Leighton tubes were exposed to sufficient virus during 1 hr at 37 C to yield a multiplicity of 20 PoFU/cell. The cells were then washed with PBS-A-γ-globulin and incubated at 37 C in medium 199-1. At intervals, the cover-slip cultures were removed from Leighton tubes, rinsed in PBS-A, drained, and fixed by immersion in ethyl alcohol precooled in glass vials to −60 C. The cover slips were then stored at −60 C or below in a Revco freezer. Cover-slip cultures processed in this fashion could be stored indefinitely. For staining, the vials were transferred from the freezer to a Dry Ice-ethyl alcohol bath. The cover slips were then removed from the alcohol, drained, and

rapidly brought to room temperature by immersion in PBS solution. The cells were then stained for 24 hr with fluorescein-labeled antibody. In some experiments, the cells were simultaneously counterstained with bovine albumin conjugated with Lissamine-Rhodamine RB-200. The stained cover slips were examined with the aid of a Zeiss fluorescence photomicroscope equipped for simultaneous phase-contrast fluorescence microscopy with an achromatic aplanatic phase fluorescence condenser (NA 1.4), a 40× oil immersion phase objective, Osram mercury burner (HBO-200), and Zeiss tungsten bulb (12 v, 60 w). The photomicrographs were taken on Kodak Panatomic-X film.

RESULTS

Description and immunological reactivity of HSV antigens in infected cells. The observation recorded in this section was made on cover-slip cultures fixed immediately after exposure to virus and at 2-hr intervals until 24 hr after infection, and then stained with the various labeled antibody preparations. These antisera made apparent in the nucleus and cytoplasm of the HSV-infected cell five distinct structures containing antigens. These were an amorphous mass quasi-filling the nucleus, large nuclear granules, small nuclear granules, a diffuse cytoplasmic fluorescence, and cytoplasmic granules. The labeled rabbit anti-infected cell debris serum illuminated all five elements. However, the nuclear fluorescence obtained with this antiserum lacked the intensity imparted by labeled Ra-BIC antibody and was not used elsewhere in this study. Instead, to facilitate characterization of the five fluorescent elements, we utilized exclusively labeled Hu-p and Ra-BIC antibody preparations. As reported previously (7), labeled Ra-BIC antibody reacts with viral antigens localized in nuclei of infected cells, i.e., the nuclear granules and the amorphous mass. The pool of human γ-globulin constituting the Hu-p reacts with viral antigens localized in the cytoplasm. Ra-BIC and Hu-p do not stain uninfected cells.

Some of the characteristics of the five compartmentalized viral antigens revealed by immunofluorescence microscopy are summarized in Table 1. The salient features of these antigens may be summarized as follows.

(i) The amorphous mass (Fig. 2) is faintly visible at 2 hr after infection. Maximal intensity is reached about 4 hr after infection, and thereafter remains unchanged. The outer surface of the fluorescent mass viewed in cross section appears to be escalloped, suggesting that it is bounded by some beadlike material which does not react with the antibody present in Ra-BIC serum (Fig. 2). The amorphous mass does not fill the nucleus entirely; there are usually two or three small bodies which remain unstained (Fig. 2A). These bodies appear to be connected to the nuclear membrane by narrow unstained processes. The identity of these nonfluorescing bodies is uncertain; early in infection they resemble nucleoli in appearance.

(ii) The large nuclear granules (Fig. 2) are localized at or near the nuclear membrane; they are irregular in shape. The average number varies from experiment to experiment from less than 0.5 to 2 granules per fluorescing nucleus; however, as many as 14 granules have been counted in a single nucleus.

(iii) The small nuclear granules are usually obscured by the fluorescence of the amorphous mass; they are best seen in preparations stained with labeled Ra-BIC antibody absorbed with virus antigen fraction S2-F or with S2-B and examined with phase-contrast fluorescence optics (Fig. 2B, 3). These granules are present in most infected nuclei; as many as 20 granules per nucleus have been counted.

(iv) The fluorescent material (Fig. 4A) described by the term "diffuse cytoplasmic fluores-

TABLE 1. *Compartmentalization, immunological reactivity, and other characteristics of herpesvirus antigen*

Antigen	Labeled antibody with which it reacts	Location	Time of appearance[a]	Time of maxima intensity[a]
Nuclear				
Amorphous mass	Ra-BIC	Fills nucleus	2–3	4–6
Large granules	Ra-BIC	Marginated	4–6	4–6
Small granules	Ra-BIC absorbed with S2-F or S2-B	Dispersed throughout nucleus	4–6	10–12
Cytoplasmic				
Diffuse	Hu-p	Fills cytoplasm	2–3	8–10
Granules	Hu-p, Hu-p absorbed with S2-F	Perinuclear	4–6	16[b]

[a] Hours after infection.
[b] Approximately.

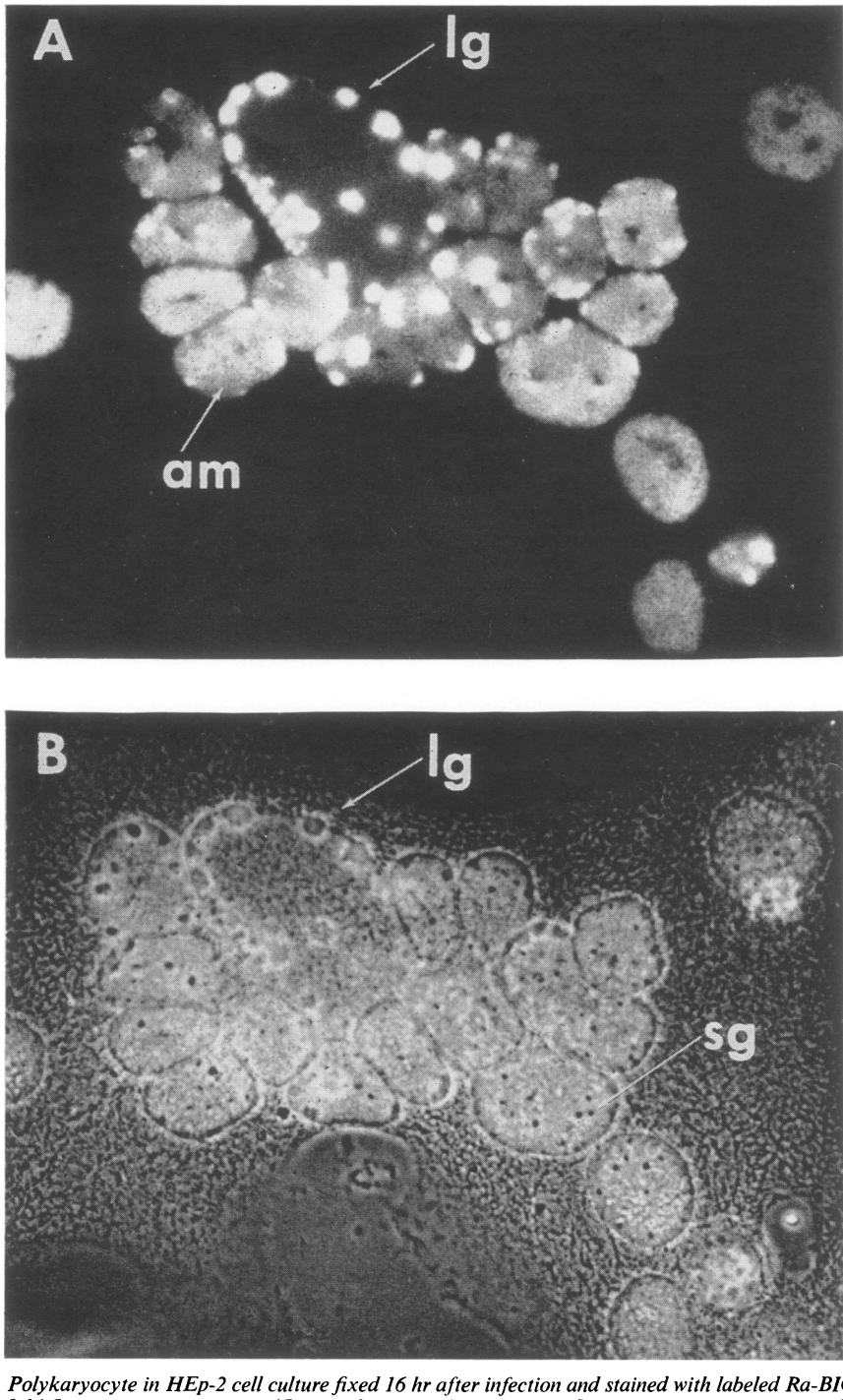

FIG. 2. *Polykaryocyte in HEp-2 cell culture fixed 16 hr after infection and stained with labeled Ra-BIC antibody. (A) Dark-field fluorescence microscopy. (B) Simultaneous phase-contrast fluorescence microscopy. Symbols: am = amorphous mass almost filling the nucleus; lg = large granules; sg = small granules seen as black dots with phase-contrast fluorescence.* × *523.*

cence" appears to consist of a powdery substance. The Hu-p antibody makes apparent in some cells in addition or in place of the powdery material a lacy network of fluorescent fibrils. There are indications that the fluorescent material consists of more than one viral antigen. Pertinent here is the observation that, whereas pool Hu-p illuminates viral antigens as early as 2 to 3 hr after infection (i.e., about the same time as Ra-BIC antibody), other pools of human antibody do not make apparent cytoplasmic viral antigens until some 4 hr after infection.

(v) The cytoplasmic granules (Fig. 4B, C) are readily seen late in infection as spherical or ovoid bodies situated at or near the nuclear membrane. In some preparations examined by phase-contrast fluorescence, the granules appeared to stick to the nuclear membrane; perhaps they were protrusions from the nucleus into the cytoplasm. The granules varied considerably in number from cell to cell. As shown in Fig. 4A, they are partially obscured by the diffuse cytoplasmic fluorescence and are therefore best seen in preparations stained with labeled Hu-p antibody absorbed with virus antigen fraction S2-F (Fig. 4B) and examined with phase-contrast fluorescence optics (Fig. 4C).

Physical properties of viral antigens. In this series of experiments, cover-slip cultures fixed 16 hr after infection were stained with conjugated Ra-BIC and Hu-p antisera absorbed with the various antigen fractions. The specific fluorescence of the various nuclear and cytoplasmic viral antigens was estimated on a scale ranging from 0 to ++++ (Table 2).

The data may be briefly summarized as follows. (i) Fractions S2-F and S2-B absorb antibodies reacting with viral antigens making up the amorphous nuclear mass; they do not absorb the antibody to nuclear granules (Fig. 3). (ii) Fraction S2-F absorbs the antibody reacting with the antigens responsible for the diffuse cytoplasmic fluorescence. Fraction S2-B does not react with this antibody. The antibody to the cytoplasmic granules is not absorbed appreciably by S2-B or S2-F (Fig. 4B, C). (iii) Fractions P1 and P2, untreated or boiled, absorb out all Ra-BIC antibody reacting with nuclear antigens. (iv) Fractions P1-F and P2-F absorb all of the Hu-p antibody reacting with diffuse and granular cytoplasmic antigens. Boiling appears to denature P1 and P2 so much that they no longer react with the antibody to either the diffuse or granular cytoplasmic viral antigens.

The results of this experiment permit two conclusions. Firstly, within the limits of the method of detection, the nuclear granules and the amor-

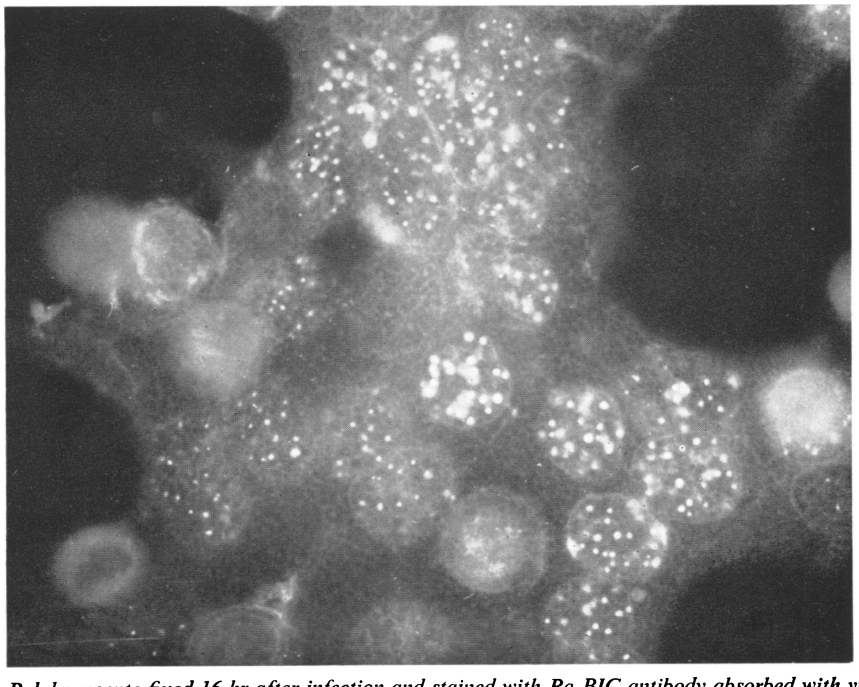

FIG. 3. *Polykaryocyte fixed 16 hr after infection and stained with Ra-BIC antibody absorbed with virus antigen fraction S2-F. Only large and small granules fluoresce. Only small granules are in focus. Dark-field fluorescence microscopy.* × *523.*

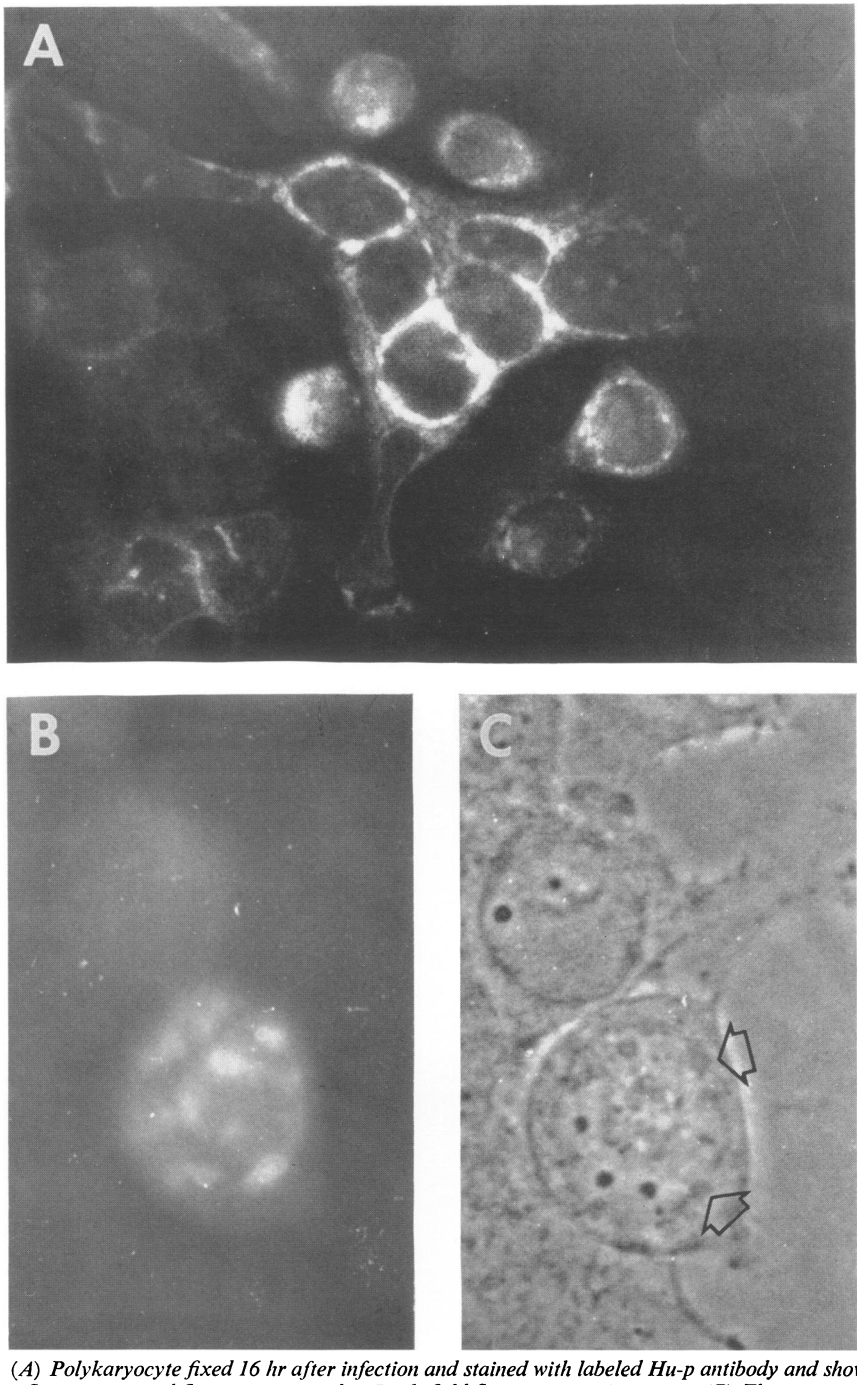

FIG. 4. *(A) Polykaryocyte fixed 16 hr after infection and stained with labeled Hu-p antibody and showing diffuse cytoplasmic fluorescence and fluorescent granules. Dark-field fluorescence microscopy. (B) Fluorescent cytoplasmic granules in cell fixed 10 hr after infection and stained with Hu-p antibody absorbed with virus antigen fraction S2-F. Dark-field fluorescence microscopy. (C) Simultaneous phase-contrast fluorescence microscopy of the same field as in B. Fluorescent granules in B appear in C (arrows) as gray perinuclear granules of moderate density. A, × 523; B and C, × 1,210.*

TABLE 2. *Immunofluorescent reactivity of labeled antibody absorbed with heated and fresh viral antigens fractionated by differential centrifugation*

Group no.	Cover-slip culture	Antibody prepn	Absorbed with[a]	Intensity of immunofluorescence			
				Nucleus		Cytoplasm	
				Amorphous mass	Granules	Diffuse	Granules
1	Infected, 10 hr	Ra-BIC	NA[b]	++++	++++	0	±
2		Ra-BIC	P1-F	+	+	0	0
3		Ra-BIC	P1-B	+	+	0	0
4		Ra-BIC	P2-F	+	+	0	0
5		Ra-BIC	P2-B	+	+	0	0
6		Ra-BIC	S2-F	+	+++	0	±
7		Ra-BIC	S2-B	+	++++	0	±
8		Hu-p	NA	0	0	++++	+++
9		Hu-p	P1-F	0	0	0	0
10		Hu-p	P1-B	0	0	++++	+++
11		Hu-p	P2-F	0	0	0	0
12		Hu-p	P2-B	0	0	++++	+++
13		Hu-p	S2-F	0	0	0	+++
14		Hu-p	S2-B	0	0	++++	+++
15	Uninfected	Ra-BIC	NA	0	0	0	0
16		Hu-p	NA	0	0	0	0

[a] See Fig. 1 for key to designation of viral antigen fractions.
[b] Not absorbed.

phous nuclear mass carry unrelated immunologically reactive sites. Similarly, the antigenic specificity of the cytoplasmic granules differs from that of the finely dispersed material responsible for the diffuse cytoplasmic fluorescence. Secondly, the antigens carrying the immunologically reactive sites of both nuclear and cytoplasmic granules sediment on centrifugation at 79,000 × *g* for 2 hr. On the other hand, there is some uncertainty about the macromolecular properties of the antigen carrying the immunologically reactive sites of the amorphous nuclear mass and of the finely dispersed cytoplasmic material, in view of the finding that the antigen is present in both the supernatant fluid and pellet obtained after centrifugation of infected cell extracts at 79,000 × *g* for 2 hr. It could be that some of the antigens in the pellet were trapped in cell debris or that they formed aggregates capable of being sedimented by the centrifugal force applied. Present evidence favors the hypothesis that more than one molecular species carry the immunologically reactive sites of the amorphous mass and of the antigen responsible for the diffuse cytoplasmic fluorescence. The conclusion is based on the observation that the material pelleted at 79,000 × *g* for 2 hr yields on rate centrifugation in a sucrose density gradient several bands of material reactive in complement fixation tests with Ra-BIC serum (Spring, *unpublished data*).

Stability of compartmentalized immunofluorescent elements to heating and to ether extraction. This experiment involved four groups, each consisting of six cover-slip cultures fixed 16 hr after infection and an equal number of uninfected, fixed cover-slip cultures. All cover slips were removed from ethyl alcohol and rinsed in PBS as described in Materials and Methods. At this point, one group was stained (three cover slips with Ra-BIC and three with Hu-p). The other three groups were heated in a drying oven at 60 C for 2 hr, 95 C for 2 hr, and shaken in ethyl ether for 3 min at room temperature, respectively, before staining. The results are summarized in Table 3.

The salient features are as follows. Nuclear and cytoplasmic granules could not be demonstrated by immunofluorescence in preparations heated at 60 C or above. The reactivity of the antigens comprising the amorphous nuclear mass with Ra-BIC antibody is reduced by heating but not by extraction with ether. Heating at 60 C or extraction with ether prior to staining did not affect the intensity of the diffuse cytoplasmic fluorescence obtained with labeled Hu-p serum (Fig. 5A). However, after heating at 95 C, the cytoplasmic antigen no longer reacted with labeled Hu-p antibody. Heating alters the immunological specificity of one or more viral antigens in that they acquire ability to react with labeled antibody. This conclusion

TABLE 3. *Effect of heating and of ethyl ether extraction on the reactivity of nuclear and cytoplasmic antigens with labeled Ra-BIC and Hu-p antibody*

Group no.	Cover slip	Treatment	Conjugated serum	Intensity of immunofluorescence	
				Diffuse cytoplasm fluorescence	Amorphous nuclear mass
1	Infected, 16 hr	None	Ra-BIC	0	++++
		None	Hu-p	++++	0
2		60 C, 2 hr	Ra-BIC	++++	++
		60 C, 2 hr	Hu-p	+++	0
3		95 C, 2 hr	Ra-BIC	++	++
		95 C, 2 hr	Hu-p	±	0
4		Ether	Ra-BIC	0	++++
		Ether	Hu-p	+++	0
5	Uninfected	None	Ra-BIC	0	0
		None	Hu-p	0	0
		60 C, 2 hr	Ra-BIC	0	0
		60 C, 2 hr	Hu-p	0	0
		95 C, 2 hr	Ra-BIC	0	0
		95 C, 2 hr	Hu-p	0	0
		Ether	Ra-BIC	0	0
		Ether	Hu-p	0	0

emerges from the observation that a diffuse specific fluorescence is observed in the cytoplasm of heated infected cells stained with labeled Ra-BIC antibody (Fig. 5B). The fluorescence is particularly intense in cells heated at 60 C and less intense in cells heated at 95 C.

Effect of arginine deprivation. It was reported by Tankersley (15) that, of all essential amino acids, the absence of arginine particularly restricts the multiplication of HSV. Two series of experiments involving arginine-deprived cells are pertinent to the studies presented here. The first shows that arginine deprivation is particularly effective late in infection. The second series shows that, in arginine-deprived cells, intranuclear and cytoplasmic granules characteristic of HSV-infected cells are not formed.

In the first series of experiments, HEp-2 cells were infected and suspended in MEM lacking arginine. The suspended cells were dispensed into four flasks and incubated with constant shaking in a water bath maintained at 34 C. Details of the procedures for infecting and suspending cells were reported elsewhere (9). Immediately after incubation sufficient arginine to yeild the previously determined optical concentration of 5×10^{-3} M was then added to flasks 1 and 2. At 4 hr after infection, the cells in flask 2 were sedimented and resuspended in MEM free of arginine. At 6 hr after infection, arginine was added to flask 4. The yield of virus obtained 15 hr after infection and suspension of cells is shown in Table 4. The results confirm Tankersley's report (15) that arginine is required for virus multiplication. The data also show that arginine is not required during the first 4 hr after infection but must be present 6 hr after infection and thereafter for virus multiplication.

In the second series of experiments, HEp-2 cells grown on cover slips were starved for 6 hr in MEM lacking arginine. The cells were then infected with virus rendered free of arginine by filtration through G-25 Sephadex and overlayered 16 cover-slip cultures with MEM containing arginine, and 16 others with medium lacking arginine. At 6 and 18 hr after infection, respectively, one-half of the cover slips in each group were fixed, stained, and examined. The results were as follows. All infected cell cover-slip cultures stained with labeled Ra-BIC and Hu-p antibody showed diffuse fluorescence and the fluorescence due to the amorphous nuclear mass, respectively. The arginine-deprived cultures could not be distinguished from controls with respect to intensity of the fluorescence. However, in cultures deprived of arginine and fixed 18 hr after infection, polykaryocytes were scarce and, moreover, both nuclear and cytoplasmic fluorescent granules were only rarely present (Fig. 6). Based on a count of nearly 4,000 cells stained with Hu-p and Ra-BIC sera absorbed with virus antigen fraction S2-F, the cultures deprived of arginine showed an approximately 30-fold decrease in the number of cells containing cytoplasmic and nuclear fluorescent granules, respectively.

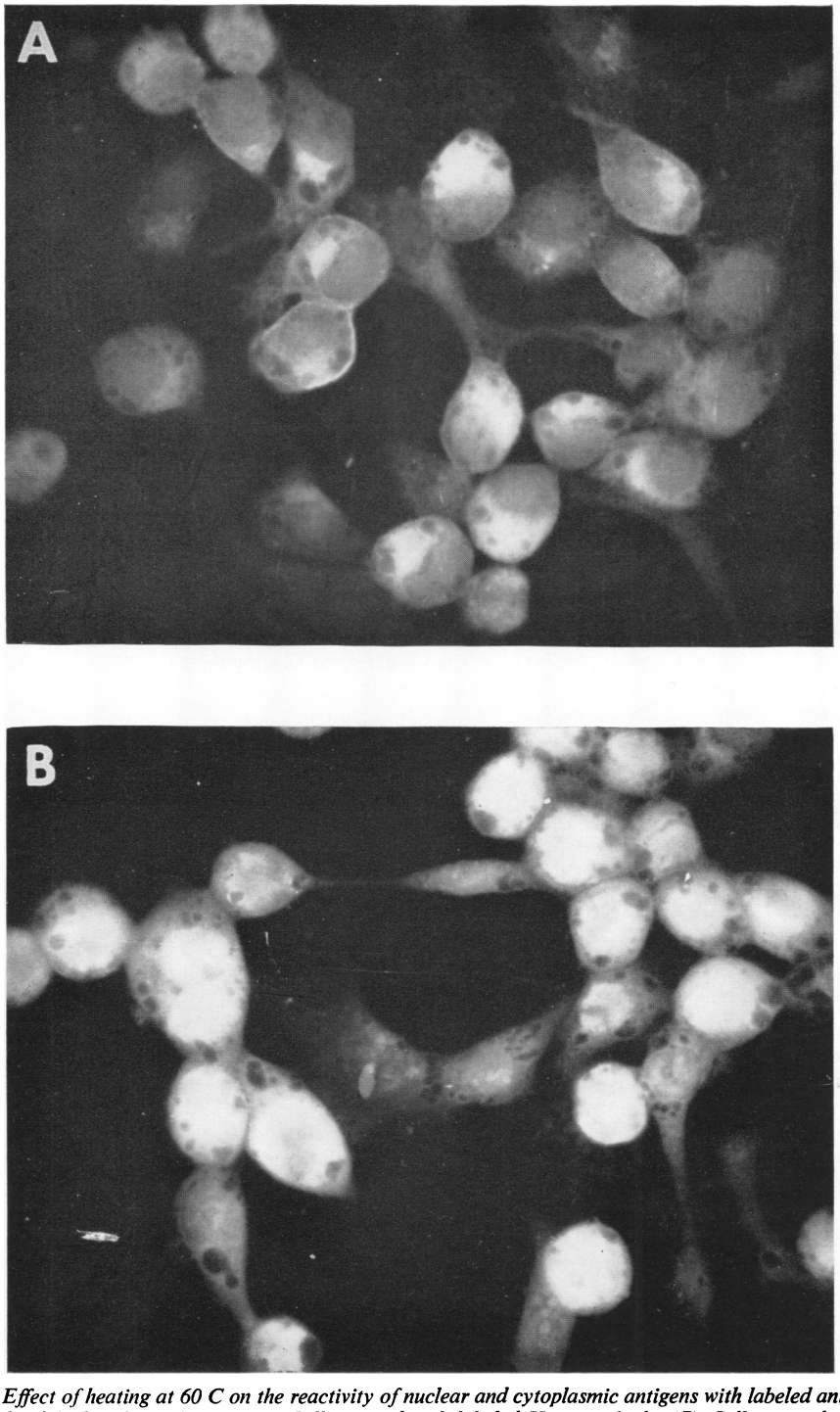

FIG. 5. *Effect of heating at 60 C on the reactivity of nuclear and cytoplasmic antigens with labeled antibody. The cells were fixed 10 hr after infection. (A) Cells stained with labeled Hu-p antibody. (B) Cells stained with labeled Ra-BIC antibody. Dark-field fluorescence microscopy. × 523.*

TABLE 4. *Virus yield from uninfected cells suspended in media deprived or enriched with arginine*

Flask no.	Arginine present	Virus yield 15 hr after infection[a]
1	0–5[b]	4.5×10^4
2	0–4	2.7×10^2
3	6–15	2.4×10^4
4	Absent	1.9×10^2

[a] Expressed as polykaryocyte-forming units per milliliter of infected HEp-2 cell suspension.
[b] Hours after infection.

DISCUSSION

Two findings are reported in this paper. The first is that the antigenic products specified by HSV in HEp-2 cells form at least five immunofluorescent elements, differing with respect to immunological specificity, physical properties, and with respect to the cellular compartment in which they localize. These elements are an amorphous mass filling the nucleus, large and small nuclear granules, cytoplasmic granules, and a powdery material dispersed throughout the cytoplasm. A sixth element appears in the cytoplasm of heated cells. The second finding of significance is that the immunofluorescent antigens are segregated in different compartments of the cells.

Immunochemical specificity of the antibody preparations. We are at loss to explain fortuitous but nevertheless restricted reactivity of the human γ-globulin preparations used in this and earlier (8) studies. Three points should be made. (i) The antibody is specifically directed against the virus, since absorption of the γ-globulin with infected cell debris abolishes its ability to neutralize virus and to stain infected cells (8). Absorption with uninfected cells was without effect. (ii) The restricted reactivity of the antibody contained in the human γ-globulin is not a general property of human antibody preparations, as evidenced by the fact that Lebrun (2), using labeled human antibody, reported intranuclear localization of HSV antigen. It should be noted parenthetically that, in a recent survey by A. B. Sabin (*personal communication*), a small fraction of human convalescent sera were found to contain antibodies reactive in complement fixation tests with boiled extracts of HSV-infected cells. (iii) The restricted reactivity of the antibody contained in the human globulin is not due to its inability to penetrate into the nucleus of fixed cells. This is evidenced by the fact that the γ-globulin pool used in the earlier study also contained antibody against measles virus. With that pool, intranuclear antigen could be readily demonstrated in measles-infected cells.

The restricted reactivity of the Ra-BIC serum is better understood. Since the rabbits were immunized with noninfectious, boiled cell debris, the antibody response was necessarily directed against (i) antigenic sites on macromolecules unaffected by heat and probably exemplified by the intranuclear antigens, and (ii) new antigenically active sites arising on denatured macromolecules and probably exemplified by the cytoplasmic antigen demonstrable only after heating.

Nature and function of the immunofluorescent elements. This discussion must take into consideration the likely hypothesis that each of the immunofluorescent elements consists of more than one antigen. This consideration restricts the interpretation of the data; nevertheless, certain conclusions can be made. The data indicate that the antigens associated with nuclear and cytoplasmic granules have the following properties. (i) They are relatively heat-labile. (ii) They are synthesized relatively late in infection. (iii) They are associated with macromolecules sedimenting at $79{,}000 \times g$ for 2 hr. (iv) They require arginine for synthesis or for assembly. These properties fit herpesvirions as well. It is noteworthy that structures similar in size to the granules described in this paper are readily seen with the aid of the electron microscope in thin sections of infected cells (Schwartz, *unpublished data*). The nature of these structures is uncertain. Pending conclusion of electron-microscopic studies now in progress, it seems probable that the granules represent viral factories and aggregates of viral constituents in different stages of assembly.

The nature and function of the antigens forming the amorphous nuclear mass and those responsible for the diffuse cytoplasmic fluorescence are less clear. Although these immunofluorescent elements are seen relatively early in infection, they could consist of both structural and nonstructural viral antigens.

The nature of cytoplasmic antigens made apparent by heating the infected cells is also uncertain. It could be that heating confers on one or more viral antigens a new immunological specificity in the same fashion that heating converts the immunological specificity of the "D" poliovirus antigen to that of the "C" antigen (10).

Cellular compartmentalization of viral antigens. The finding that Hu-p and Ra-BIC antibodies illuminate antigens in different compartments of the cell raises two obvious questions, namely, how and why are viral antigens segregated in different compartments of the cells.

Fundamentally, two hypotheses could explain our observations. Hypothesis 1 is that antigenic products specified by the virus are synthesized in either the nucleus or cytoplasm and that the anti-

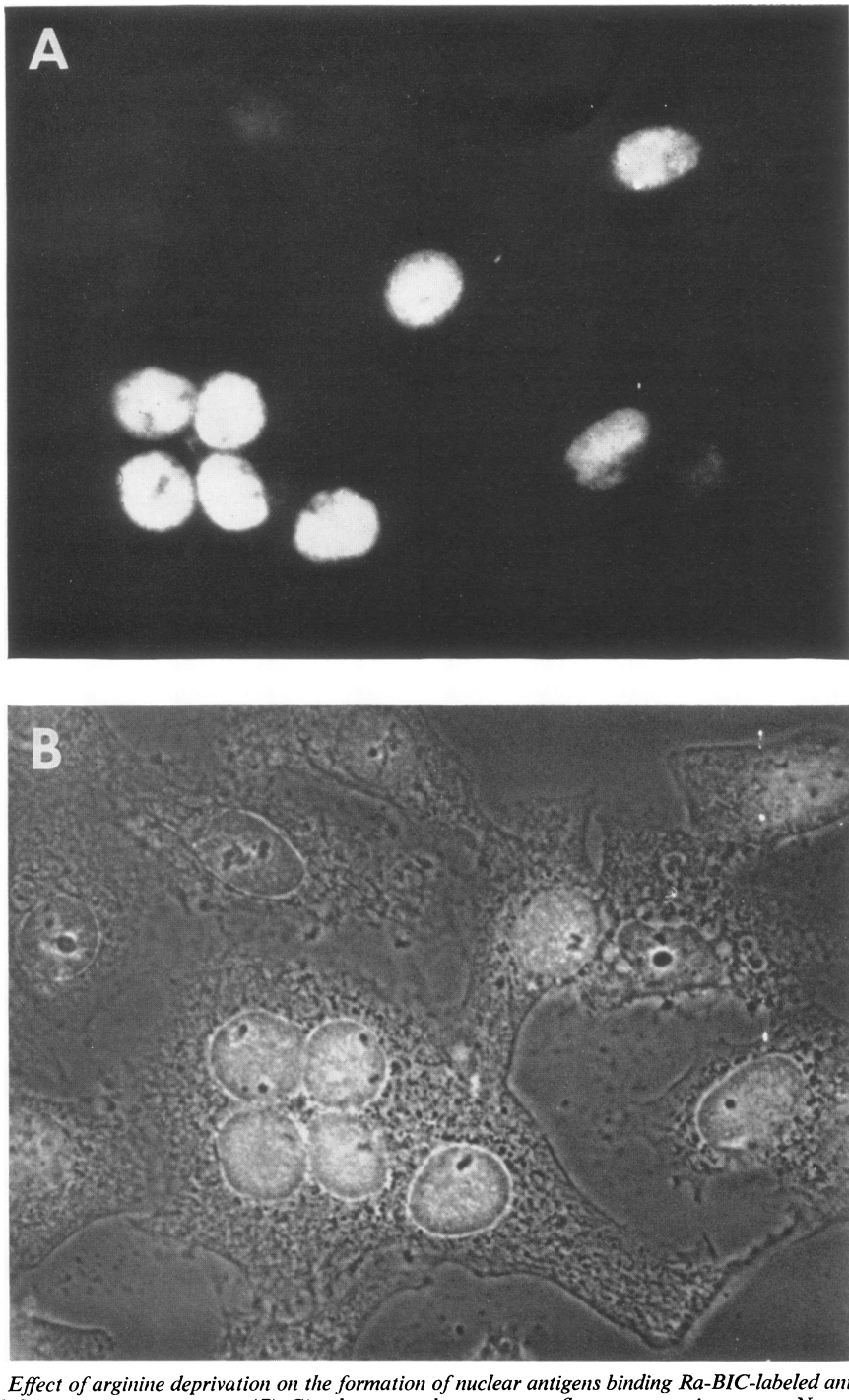

FIG. 6. *Effect of arginine deprivation on the formation of nuclear antigens binding Ra-BIC-labeled antibody. (A) Dark-field fluorescence microscopy. (B) Simultaneous phase-contrast fluorescence microscopy. Note absence of fluorescent nuclear granules. The irregular dark nuclear bodies in B are present in uninfected cells as well and correspond to the nonfluorescent nuclear structures described in the text.* × *523.*

gens accumulate in the compartment in which they are made. Hypothesis 2 is that all antigens are made in one compartment but are selectively transported or become immunologically altered as they cross into the other compartment. We do not have sufficient data to discriminate between these hypotheses, nor are they mutally exclusive. However, present evidence tends to favor the second hypothesis. Thus, recent studies in this laboratory (14) suggest that the bulk of viral proteins made after virus infection are synthesized on cytoplasmic polyribosomes. On the other hand, electron microscopic (4, 5) and biochemical studies (Sydiskis and Roizman, *unpublished data*) indicate that the synthesis of viral deoxyribonucleic acid and assembly of the virion take place in the nucleus. It could be that the transport of viral protein from cytoplasm to the nucleus is selective, or that the protein acquires a new immunological reactivity in the process.

The questions posed here do not uniquely concern themselves with cellular compartmentalization of HSV-directed products, but rather with the mechanisms by which viruses in general multiply in eukaryotic cells. It is not clear what factors determine whether a virus will multiply in the cytoplasm or in the nucleus, how the virus reaches efficiently the site in which it will multiply, and, lastly, how it effects the transport of its constituents from one cellular compartment to another.

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