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Isolation and Characterization of a Herpes-like Virus from New Zealand Albino Rabbit Kidney Cell Cultures: a Probable Reisolation of Virus III of Rivers

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An agent which possesses the physical, chemical, cytopathic, histological, and electron microscopic attributes of a herpes group virus was isolated from an uninoculated batch of primary rabbit kidney cell cultures. Preliminary evidence indicates that antibodies against the agent are found in some sera of other "normal" New Zealand albino rabbits. In cell cultures, the virus grew best and almost exclusively in cells of rabbit origin. On the basis of these facts, the name herpesvirus cuniculi (HC) is suggested for the isolate. A batch of anti-herpesvirus bovis antiserum prepared in rabbits was found to be "contaminated" with unsuspected neutralizing antibodies against HC. Caution is mandatory when using rabbits, rabbit tissues, or rabbit sera for work with any herpes group virus unless precautions are taken to rule out unsuspected infection with or antibodies against HC. This agent may well represent a reisolation of virus III, a rabbit herpes virus, described by Rivers in 1923; the isolation of this virus has not been reported since 1940. It is important to reemphasize the existence of this agent in an animal which is commonly used for laboratory investigation of herpes group viruses.

A slowly progressing cytopathic effect (CPE) noted in newly prepared primary rabbit kidney (PRK) monolayer cell cultures led to the isolation of a viral contaminant. In this paper, certan characteristics of this agent are defined which indicate it is a member of the herpes group of viruses. Its relationship to a previously described herpesvirus of rabbits, virus III of Rivers (26, 27), is discussed.

MATERIALS AND METHODS

Cell cultures. Primary rabbit cell cultures (kidney, lung, salivary gland, and testis) were made from organs removed from 10-week-old New Zealand albino rabbits and were prepared by trypsinization essentially according to the method of Youngneer (31). Cell growth was initiated with Hanks' balanced salt solution (HBSS) containing 0.5% lactalbumin hydrolysate and 5% heat-inactivated calf serum. After a monolayer was established, cultures were maintained by weekly changes of basal medium Eagle (BME) supplemented with 10% fetal calf serum (FCS), 10% medium NCTC-109, and glutamine. (Unless otherwise indicated, all media and sera were obtained from Grand Island Biological Co.), Grand Island, N.Y. This medium keeps PRK monolayers in excellent condition for many weeks and requires changing only once every 7 to 10 days.

Chick embryo (9 day) fibroblast (CEF) and whole rabbit embryo (17 day) cultures were prepared by standard methods used for CEF (28), with BME supplemented with 2% FCS and glutamine for both growth and maintenance of the monolayers.

Primary human amnion was prepared as described by Milovanovic et al. (21). The growth and maintenance medium which was replenished weekly consisted of 45% HBSS, 45% bovine amniotic fluid, 5% beef embryo extract, and 5% heat-inactivated horse serum (10).

Cultures of the continuous African grivet monkey kidney line GMK-AH-1 (12) were grown and maintained on Puck's N-16 medium supplemented with 10% FCS, 10% heat-inactivated horse serum, 10% NCTC-109, and glutamine.

All other cell cultures were purchased from Flow Laboratories, Rockville, Md., and were maintained on BME with the addition of 10% FCS and glutamine. All media contained 200 units of penicillin, 200 μg of streptomycin, 50 μg of amphotericin B, and sufficient 7.5% sodium bicarbonate to adjust the *p*H to 7.2 to

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7.5 units. Unless otherwise specified, all cultures were fed twice weekly.

Egg inoculation. Chorioallantoic membrane inoculation of embryonated hens' eggs was performed according to standard techniques (17).

Virus. Experiments were carried out with virus from the fifth to the eighth PRK passage of the isolate described. Stock virus was prepared by subjecting monolayers showing 75% CPE to three cycles of rapid freezing at −70 C and rapid thawing at 37 C. This material was pooled and centrifuged at 1,000 × *g* for 10 min; then the supernatant fluid was dispensed in small portions (with or without the addition of equal parts of sterile skim milk) and was stored at −70 C.

Virus titration. Virus titrations were carried out by inoculating 0.1 ml of serial 10-fold dilutions of virus into PRK cell cultures which were then incubated at 36 C in a stationary position. End points were determined after 24 days of incubation by the appearance and progression of typical CPE. The 50% infective dose ($TCID_{50}$) was determined by the method of Reed and Muench (24). Determination of infectious titer by plaque count with agar, methylcellulose, or antibody-containing medium was attempted, but readable plaques were never obtained.

Neutralizations. Twofold dilutions of heat-inactivated (30 min at 56 C) test sera were incubated with equal volumes of virus suspension for 3.5 hr at 4 C and 0.5 hr at 37 C; then 0.1 ml of the test mixture was inoculated into each of three PRK monolayers. Because the agent produces very sparse and slowly progressing CPE at low absolute titers, a challenge dose of virus of 100 $TCID_{50}$ determined by reading the experiments at 12 days (approximately 10,000 $TCID_{50}$ based on 24 days titers) was used. This test system produced faster, more reliable, and more easily readable neutralization end points. All neutralization end points reported are for that dilution of serum which at 12 days completely inhibited the CPE of the challenge dose of virus. Partial inhibition of viral CPE was present for two or more dilutions beyond the end point reported. Tubes containing just enough antiserum to completely inhibit CPE at 12 days showed viral CPE when the tubes were reread at 24 days, but tubes containing higher concentrations of antiserum completely suppressed CPE.

Antiserum. Antisera against the isolate were prepared by injecting rabbits and guinea pigs with $10^{4.5}$ $TCID_{50}$ of virus subcutaneously at biweekly intervals. Two weeks after the third injection, animals were bled and the serum was frozen in small samples at −20 C. Horse antiserum against herpes simplex virus was obtained from the Communicable Disease Center, Atlanta, Ga. High titer antisera against the sand rat nuclear inclusion agent (19) and against herpesvirus suis, canis, bovis, and tamarinus were kindly supplied by Luiz Melendez of the Harvard Primate Research Center, Southboro, Mass.

Staining procedures. Hematoxylin and eosin staining of monolayer cultures was carried out after fixation in Bouin's solution for 18 hr by the method of Enders and Peebles (11). Photos were taken with the Zeiss photomicroscope with Adox KB14 or Kodachrome II professional type A film. Acridine orange staining was performed on cover slip cell cultures fixed in Carnoy's solution for 1 hr according to the method of Dart and Turner (9). Digestion of deoxyribonucleic acid (DNA) was carried out by incubation of fixed monolayers with once crystallized 0.1% deoxyribonuclease in 0.02 M tris(hydroxymethyl)aminomethane buffer (*p*H 7.3) containing 0.003 M $MgCl_2$ for 1 hr at 37 C. Control monolayers were incubated in buffer.

Methyl green pyronin staining was kindly performed by A. Cohen of the Massachusetts General Hospital according to the method of Taft (29). Immunofluorescence studies were carried out by the indirect method. Infected PRK cover slip cultures were washed twice with 0.1 M phosphate-buffered saline (PBS; *p*H 7.2), fixed in acetone for 10 min at 4 C, air-dried, and stored at −70 C until used. The fixed monolayers were covered with diluted (1:10 in PBS) guinea pig antiserum against the isolate for 1 hr at 37 C, washed three times with PBS, and stained with fluoroscein-labeled goat anti-guinea pig gamma globulin (Sylvana Co., Millburn, N.J.) for 1 hr at 37 C. Cover slips were then washed twice in PBS and mounted in PBS with 10% glycerin.

Specimens stained with acridine orange and fluorescent antibody were examined with a Zeiss fluorescent microscope equipped with a 4-mm BG12 exciter filter and no. 50 and no. 44 barrier filters.

Electron microscopy. Electron microscopic studies were kindly performed by Toichiro Kuwabara, Howe Laboratory of Ophthalmology, Harvard Medical School. For negative staining, PRK monolayers showing 75% CPE were harvested and prepared as described by Plummer and Waterson (23). For positive staining, infected PRK monolayers were washed three times in PBS and were fixed for 20 min at 25 C in 4% glutaraldehyde and for 90 min at 4 C in 1% osmium tetroxide. Tissues were dehydrated in a graded series of alcohols, embedded in epoxy resin (Epon 812), sectioned, stained with uranyl acetate and lead acetate, and examined with a Jeolco electron microscope (16).

RESULTS

Isolation. Approximately 2 weeks after the preparation of primary rabbit kidney monolayer cell cultures from an apparently healthy 1-kg female New Zealand albino rabbit, scattered rounding and loss of cells were noted in all tubes (Fig. 1 to 3). Fluid harvested from these uninoculated tubes produced similar slowly progressing CPE in other noncontaminated batches of PRK monolayer cell cultures. The isolate, designated 923J, has now been propagated through 15 serial passages in PRK.

Culture characteristics. Agent 923J grew best in PRK, in which it produced CPE in 36 hr when introduced in very high titers (10^7 $TCID_{50}$). With the usual inoculum of 0.1 ml of undiluted culture fluid (10^6 $TCID_{50}$), CPE appeared at 3 to 5 days, progressing to complete destruction of the tissue sheet in 12 to 14 days. At low concentrations (1 to 10 $TCID_{50}$) beginning CPE could be detected in 13 to 17 days and progressed slowly to full monolayer involvement in 25 to 35 days. Har-

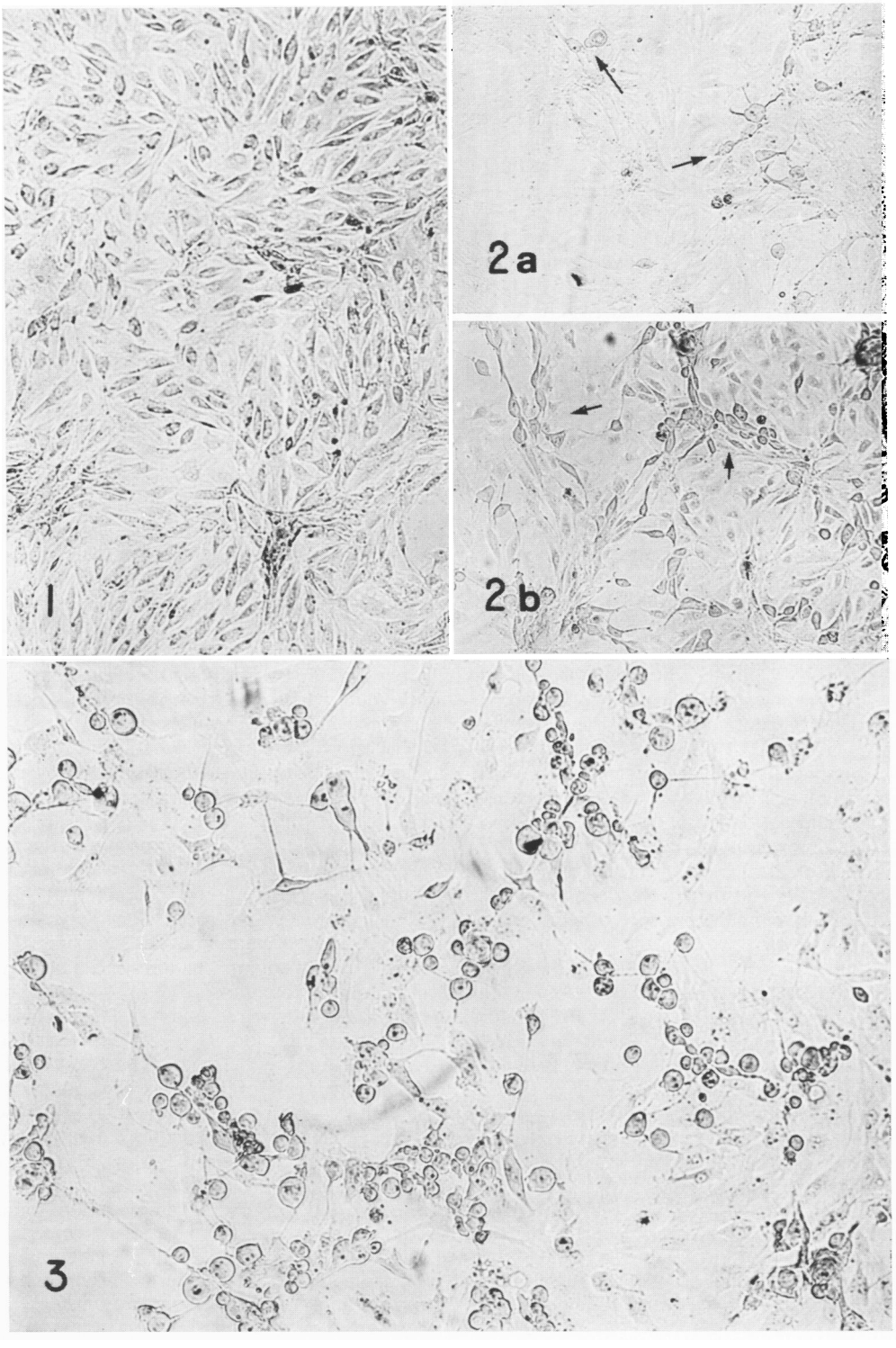

FIG. 1. *Normal rabbit kidney cell culture, unstained.* × *180.*

FIG. 2. *(a) Early cytopathic effect of 923J(HC) in PRK. (b) The changes are difficult to differentiate from "starvation" or degeneration. Unstained.* × *120.*

FIG. 3. *Moderately advanced cytopathic effect in PRK, unstained.* × *220.*

vested] when 75% of the monolayer was involved, the virus yielded titers of $10^{3.5}$ to $10^{6.5}$ $TCID_{50}$/0.1 ml when tested in PRK and read at 24 days.

When PRK monolayers were allowed to grow for 10 days or more without being refed with fresh medium, scattered rounding of cells resembling early CPE of 923J developed. This "starvation effect" was reversed within 24 hr by addition of fresh medium. Virus-induced CPE was not altered by changes of medium. To prevent confusion, cultures were fed at weekly intervals and within 48 hr of final reading of experiments.

All rabbit cell cultures tested supported growth of the virus, showed typical CPE, and exhibited Cowdry type A intranuclear inclusions (8; Fig. 4). The rabbit tissues tested were RK-13 and SIRC continuous lines, in addition to the following primary tissues: whole embryo, kidney, lung, salivary gland, and testis. Fluid from these infected cultures produced typical CPE in PRK.

There were 16 types of monolayer cultures which were not of rabbit origin and which did not support the growth of 923J when 10^4 $TCID_{50}$ were inoculated. These cultures were human amnion, human embryo kidney, human embryo lung, human kidney, Chang conjunctiva (continuous lines), HeLa (continuous line), HEp-2 (continuous line), KB (continuous line), rhesus monkey kidney, GMK–AH-1 (continuous line), LLC–MK2 (continuous line), Salk monkey heart (continuous line), beef embryo kidney, canine kidney, newborn hamster kidney, and chick embryo fibroblast. One month after infection, none of these cultures showed CPE or intranuclear inclusions. Passage of cells and fluid from these cultures to PRK failed to show the presence of infectious virus.

The only nonrabbit tissue to support the growth of 923J was primary African grivet monkey kidney (AGMK). When 10^4 $TCID_{50}$ were inoculated, very slowly progressing CPE was noted at 15 to 18 days. When tested 1 month after inoculation, AGMK cultures demonstrating CPE and intranuclear inclusions contained infectious virus in low titers as determined by passage to PRK. However, the virus produced in AGMK did not infect fresh AGMK cultures. Whether this lack of reinfecting ability for AGMK was related to a low titer of input virus or to some change in the virus was not determined.

Pathogenicity. Attempts to produce pocks on the chorioallantoic membrane of embryonated hens' eggs (CAM) were unsuccessful. Repeated intramuscular and subcutaneous injection of $10^{4.5}$ $TCID_{50}$ produced no visible effect in rabbits or guinea pigs. After such injections, neutralizing antibodies were detected in the serum. Neutralizing antibody levels obtained in rabbits were consistantly higher than those obtained in guinea pigs. The highest rabbit titers were 1:1,032 as compared to 1:32 in guinea pigs.

Infection of abraded rabbit cornea produced by instilling $10^{4.5}$ $TCID_{50}$ of virus in the conjunctival cul-de-sac caused a mild superficial punctate keratitis without conjunctivitis, iritis, or encephalitis. No dendritic figures were observed.

Physical characteristics. Tests were conducted with virus at a concentration of $10^{3.5}$ $TCID_{50}$/0.1 ml in BME with 10% FCS.

At 25 C, there was loss of 1 log of infectious virus per 24-hr period. After 96 hr at this temperature, no infectious virus was detected. At 45 C, the titer dropped approximately 1 log unit per hr, whereas at 55 C the virus was completely inactivated in 10 min.

The loss of infectivity was approximately the same at 4 C as it was at 25 C, about 1 log per 24 hr. In the initial period of storage at −70 C, the virus was relatively stable; the titer dropped 0.5 log in 5 months. However, the same lot of virus, tested after 1 year of storage at −70 C, showed a loss of 3.0 logs of infectious titer. Virus has been recovered in low titer from samples frozen for 2.5 years. Mixing the virus suspension with equal parts of sterile skim milk did not enhance the viability of samples stored at −70 C.

The agent was relatively stable after three cycles of freezing at −70 C, followed by rapid thawing at 37 C; there was a drop in titer from $10^{5.5}$ to $10^{4.7}$ $TCID_{50}$.

Chemical characteristics. Exposure of the virus suspension ($10^{3.5}$ $TCID_{50}$/0.1 ml) to diethyl ether (four parts suspension to one part ether) at 4 C for 18 hr completely destroyed infectivity. In the presence of 10^{-4} M 5-iodo-2′-deoxyuridine (IDU) added to the medium 3 hr after infection, a few cells showed rounding at 4 to 6 days. These changes did not progress and no CPE was evident 3 weeks after infection. Passage of cells from IDU-treated cultures at the end of 2 weeks failed to reveal the presence of infectious virus. In contrast, the cultures maintained without IDU showed early CPE in 6 days and complete involvement of the tissue sheet in 18 days.

Cytochemical characteristics. Staining of infected cover slip preparations with acridine orange revealed cytoplasmic DNA-staining material in cells showing advanced CPE. Inclusions corresponding to the intranuclear inclusions in hematoxylin- and eosin-stained preparations gave intense DNA-type staining. This staining was not observed in preparations pretreated with deoxyribonuclease. Similar results were obtained with the methyl green pyronin staining technique.

Fluorescent antibody staining with the indirect method showed intense staining of the cytoplasm, nucleus, and intranuclear inclusions of infected cells. Such staining was not present in control cultures.

Hematoxylin and eosin staining performed on monolayers fixed at different intervals after infection provided information about the morphogenesis of intranuclear inclusions and about the relationship of histological changes to CPE. At 48 hr after infection (10^4 $TCID_{50}$), neither CPE nor abnormalities of the stained cells were evident. At 72 hr, no CPE was seen, but in stained preparations a rare cell showed signs of viral infection (margination of chromatin or early intranuclear inclusion formation). With this titer of input virus, it was not until 96 hr that the earliest signs of CPE (hyperrefractility and rounding of scattered cells) were recognizable. However, the effect of viral infection in stained preparations as well developed, with approximately one-fourth of the cells showing some stage of intranuclear inclusion formation. It was evident that in the living monolayer many cells which appeared normal actually possessed moderately advanced intranuclear inclusions. Figure 4, a stained monolayer fixed at 120 hr after infection, shows cells with intranuclear inclusions in various stages of development and illustrates, in one picture, the sequence of events ascertained from examination of many specimens. A constant finding was that a cell exhibiting unequivocal CPE, when stained, showed scant dark-staining cytoplasm and a pyknotic nucleus containing an advanced intranuclear inclusion. A number of small multinucleate giant cells, containing three to seven nuclei, were seen. Each nucleus contained a well-developed intranuclear inclusion, whereas the surrounding cytoplasm was invariably scant and abnormally basophilic.

The histological changes and intranuclear inclusions produced by 923J were indistinguishable from changes produced by herpesvirus hominis and other viruses of the herpes group (5, 15).

Electron microscopic appearance. Negatively stained preparations contained particles of the shape, size, and configuration described for other herpes group viruses (15, 23; Fig. 5 and 6). There were multiple hollow-appearing capsomeres on the surface of particles which were 0.11 to 0.12 μm in diameter. The resolution of the micrographs did not allow a definite capsomere count or determination of symmetry.

In positively stained thin sections of infected PRK monolayer cells, particles indistinguishable from other members of the herpes virus group (7, 15, 18; Fig. 7 to 10) were seen. Cytoplasmic and extracellular virus particles had a rough surfaced outer coat measuring 0.150 to 0.170 μm in diameter. Within this layer, one could discern a structure similar to the uncoated virus particles seen in the nucleus; that is, a wall about 0.070 to 0.075 μm in diameter surrounding a clear space which frequently contained an eccentric nucleoid, oblong in shape, measuring 0.048 by 0.060 μm.

Serological characteristics. Antisera produced by repeated injections of guinea pigs or rabbits with the agent gave reliable neutralization reactions in vitro. Five high-titer antisera against various other herpes group viruses failed to neutralize 923J (Table 1). The only exception was a rabbit antiserum against herpesvirus bovis, which, when diluted 1:128, neutralized 923J completely. However, herpesvirus bovis antiserum of bovine origin failed to neutralize 923J, and high-titer anti-923J serum did not neutralize herpesvirus bovis. Recently, Melendez (*unpublished data*) has found that normal rabbits in his colony had serum neutralizing activity against 923J. Taken together, these data suggest that the sample of herpesvirus bovis antiserum which was produced in rabbits was "contaminated" with unsuspected neutralizing antibodies against the rabbit herpesvirus 923J.

DISCUSSION

Agent 923J, an isolate obtained from uninoculated PRK monolayer cultures, possesses the physical, chemical, cytopathic, histological, and electron microscopic characteristics of a herpes group virus. Since two other lots of PRK, made and maintained with the same batch of reagents, showed no signs of viral CPE and since PRK monolayers tested to date have not varied significantly in their susceptibility to infection with the agent, it follows that the virus was actually present in the rabbit tissue used to prepare the "contaminated" cultures. The latter facts, taken together with the agent's in vitro predilection for rabbit tissues and the evidence that antibodies, against it can be found in sera of normal rabbits, strongly suggest that this herpesvirus is truly native to the rabbit.

Based on the data presented, it is suggested that the isolate be named herpesvirus cuniculi (HC). This is the name that was subsequently given to a rabbit agent (virus III) isolated in 1923 (26). Curiously, no isolation of virus III has been reported since 1940 (5). [In 1960, the late J. M. Pearce (22) reported the results of experiments with virus III. No information is available regarding the source of the virus and the actual dates when these experiments were performed.] The relationship of the present isolate (HC) to virus III is discussed below.

In 1923, Rivers and Tillett (26) attempted to

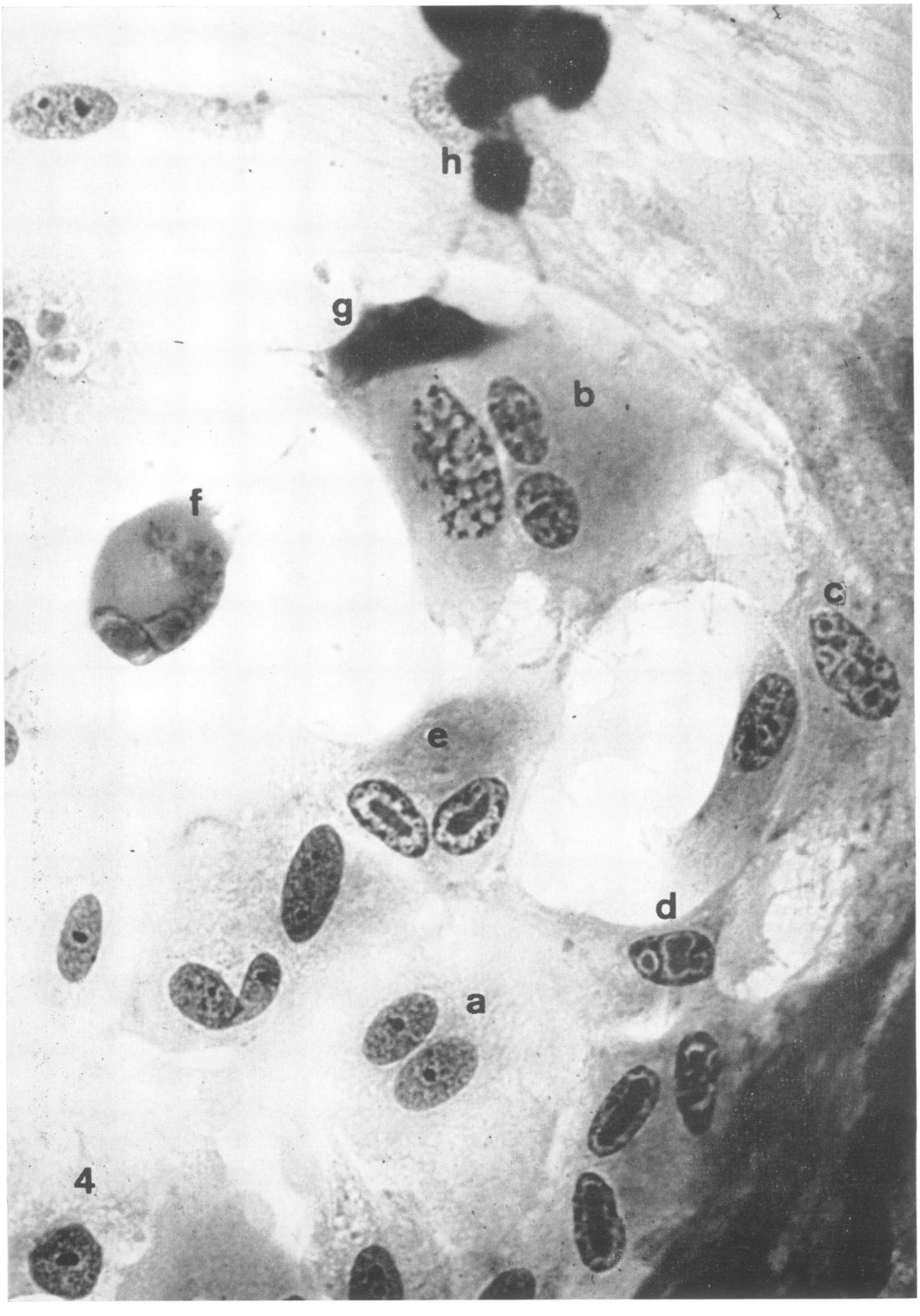

FIG. 4. *PRK fixed at 120 hr after infection, illustrating the morphogenesis of intranuclear inclusions with 923J(HC). (a) Binucleate normal cell; (b) early intranuclear inclusion formation with beginning disappearance of nucleoli and increased coarseness of chromatin; (c) many small intranuclear inclusions; (d) coalescence of smaller inclusions; (e) moderately advanced inclusion with cells showing little change which would be interpreted as CPE in living monolayer; (f) small multinucleate giant cell, the nuclei of which contain advanced inclusions whereas the cytoplasm is deeply basophilic; (g and h) these cells, which correspond to those showing advanced CPE in the unstained specimen, stain so basophically that no details are recognizable in photo. Each cell contains an advanced intranuclear inclusion. Hematoxylin and eosin staining.* × *420.*

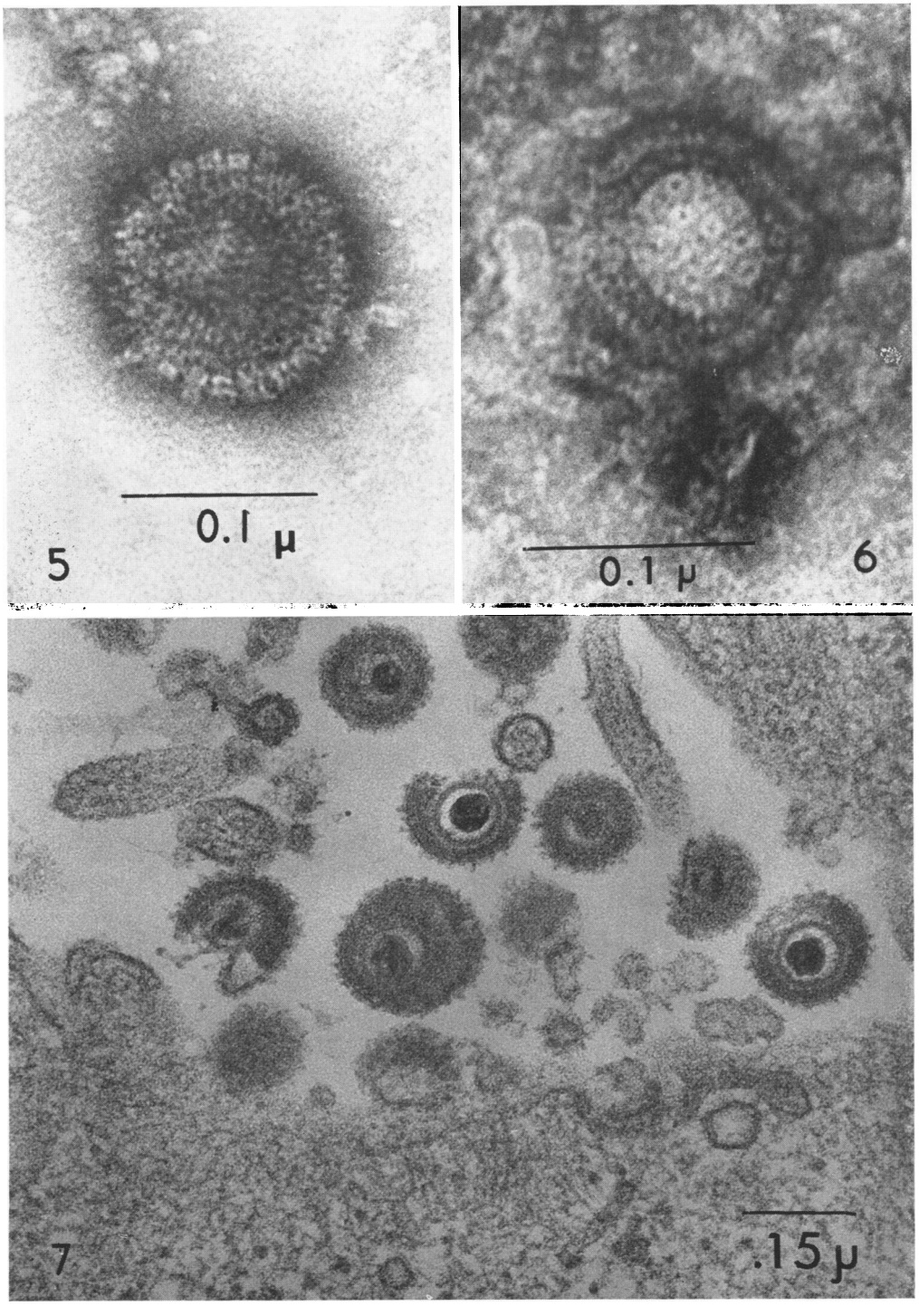

FIG. 5. *Negatively stained 923J(HC) particle.* × *276,000.*
FIG. 6. *Negatively stained 923J(HC) virus particle showing hollow capsomeres.* × *340,000.*
FIG. 7. *Thin-section electron micrograph showing a number of fully developed and enveloped extracellular herpes-type virus particles.* × *108,000.*

find the etiologic agent of varicella by serial passage of patient's blood in rabbit testicle. After a few passages, they isolated an agent which they called virus III; in rabbits, this agent produced fever, an exanthem, skin vesicles, corneal lesions, and intranuclear inclusions, very reminiscent of herpes zoster-varicella infection in humans. Seven months later, the authors were forced to recant their claim of having isolated the causative agent of varicella (27). They found that 20% of uninoculated rabbits possessed neutralizing antibodies against virus III and that convalescent sera from patients with varicella or herpes simplex infections failed to inactivate their isolate.

In 1924, Miller, Andrewes, and Swift (20) accidentally isolated a similar virus. They acquired evidence that virus III was of rabbit origin by isolating an agent indistinguishable from virus III from serially passaged "normal" rabbit testicular tissue (6).

Isolation of a similar agent was reported by McCartney in England (1) and by Doerr in Switzerland (30). Estimates of the prevalence of infection, as judged by resistance to experimental infection, varied markedly. Andrewes (1), in Hampstead, England, found 2.1% of 377 rabbits resistant; Rivers, in New York, estimated that 15% of his stock rabbits were resistant; and Topacio and Hyde estimated that 17% of Maryland rabbits were resistant (30).

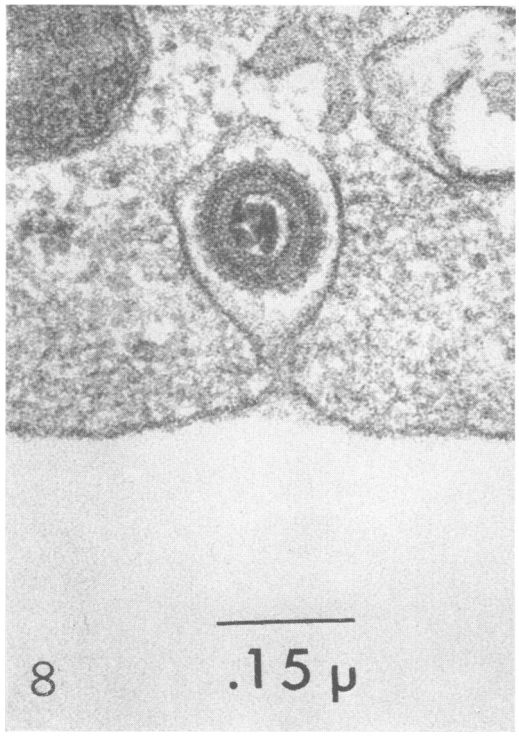

FIG. 8. *Thin section showing a virus particle which appears to be entering the cell in a pinocytotic vesicle. Morphological appearance is consistent with classification as a herpesvirus.* × *123,000.*

Normally, the virus was recoverable from a freshly infected animal for only a short period (7). However, Rivers and Pearce (25) and Andrewes (4) showed that virus III could produce chronic infection of transplantable rabbit tumors. An additional interesting observation was made by Andrewes (4); he found that, when virus III was "in excess in mixtures with fibroma virus, it suppressed the skin lesions of the latter altogether."

The tissue culture characteristics of virus III were reported by Andrewes in 1929 (2, 3), by Topacio and Hyde in 1932 (30), and by Ivanovics and Hyde in 1936 (14). All rabbit tissues that they grew in vitro exhibited intranuclear inclusions when infected with virus III. No data are available about the in vitro susceptibility of nonrabbit tissues to infection with virus III. However, studies in vivo showed that rats, guinea pigs, monkeys, and the CAM of embryonated hens' eggs were not affected by the virus (30). Pearce (22) reported valvular lesions in rabbits given the virus intravenously.

A comparison between certain tests carried out on HC in this study and similar tests reported in the literature on virus III is presented in Table 2. Because many attributes of virus III described in these papers were nonspecific, no attempt was made to repeat all of the experiments which had been carried out on that agent.

In essence, both HC and virus III produce an asymptomatic infection in rabbits, CPE and Cowdry type A intranuclear inclusions in all rabbit cell cultures, and no visible disease in guinea pigs or on the CAM; they also possess a similar resistance to heat inactivation. In all properties examined, the viruses are similar. More rigorous proof of identity of these viruses depends upon reciprocal serological tests. Unfortunately, attempts to procure samples of virus III or its antiserum from a number of sources have met with failure. Consequently, the question of whether the present isolate and virus III are identical or are representatives of closely related viruses cannot be answered.

The present investigation leaves many questions to be answered. What is the current prevalence of HC infection in various rabbit populations? What is its mode of transmission and its distribution in the infected rabbit? Were the rabbit kidney cells from which it was isolated harboring a chronic inapparent infection or was it by chance that the kidney was removed for

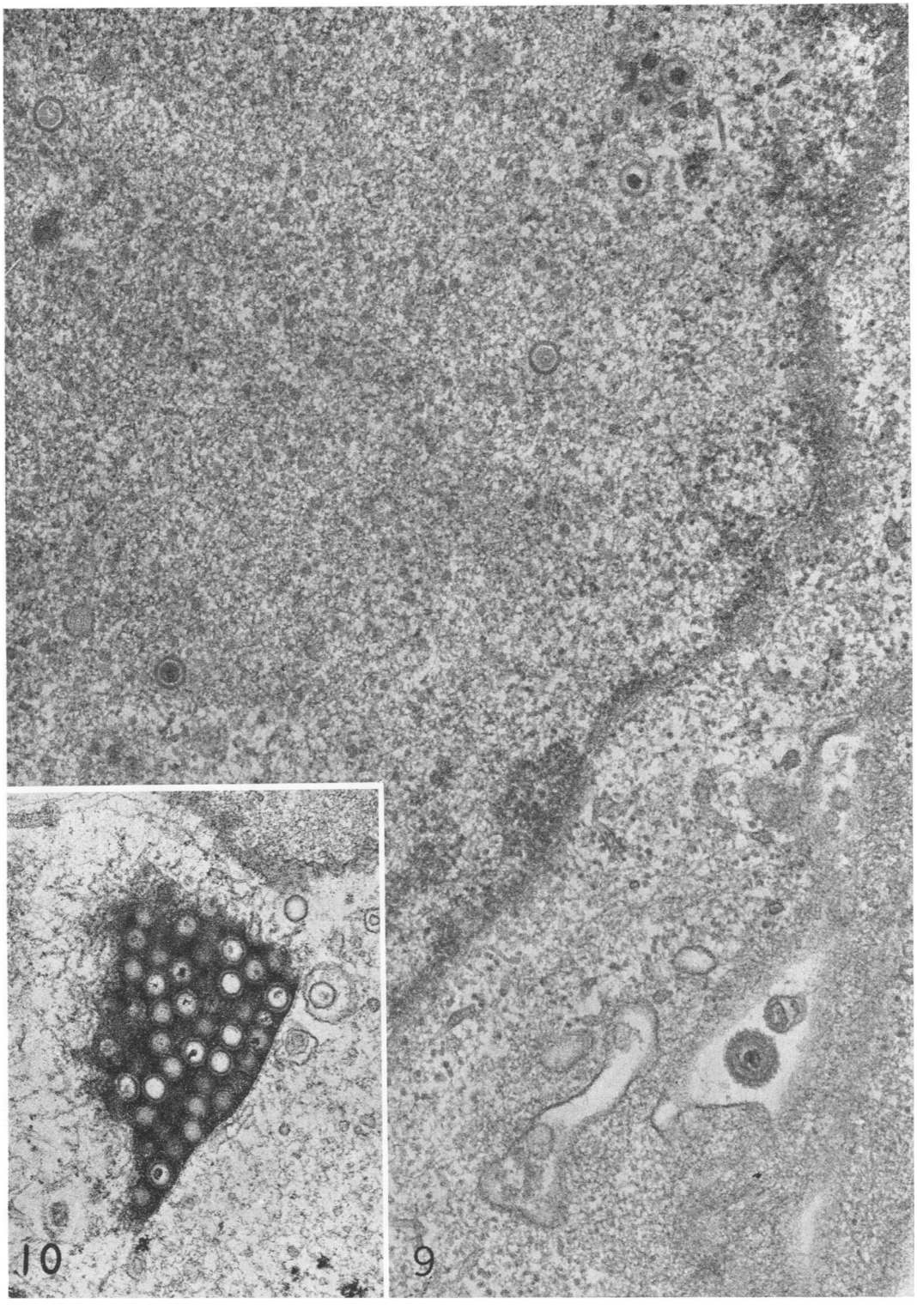

FIG. 9. *Cell nucleus shows many single membrane forms of the virus. Those clustered in the upper right hand corner appear to form a primitive lattice of virus particles. An enveloped particle is seen in an extracellular cleft within the cytoplasm.* × *54,750.*

FIG. 10. *Crystalline lattice of single membrane particles. This lattice which is normally seen in the nucleus was found in the cytoplasm of a cell showing advanced degeneration and disorganization.* × *22,200.*

TABLE 1. *Serological characteristics of 923J (herpesvirus cuniculi)*

Antiserum[a]	Source	Neutralization index vs. homologous agent[b]	Dilution	Neutralization activity vs. 100 $TCID_{50}$[c] (923J)
Herpesvirus cuniculi (923J)	Guinea pig		1:32	Incomplete
Herpesvirus cuniculi (923J)	Rabbit		1:128	Complete
Herpesvirus bovis	Rabbit	4.0	1:128	Complete
Herpesvirus bovis	Bovine	4.5	1:8	None
Herpesvirus hominis	Rabbit	4.5	1:8	None
Herpesvirus suis	Rabbit	3.5	1:8	None
Herpesvirus tamarinus	Rabbit	3.5	1:8	None
Sand rat herpes virus[d]	Rabbit	5.5	1:8	None

[a] All antisera except those against H. cuniculi were kindly supplied by Dr. Luis Melendez.
[b] Neutralization index was performed on rabbit kidney with the serum diluted 1:3 (19).
[c] Based on 12 day titer in PRK.
[d] *See* reference 19.

TABLE 2. *In vitro and in vivo characteristics established for both virus III and 923J (HC)*

Characteristic	Virus III (7)[a]	923J (HC)
In vitro		
Growth in all rabbit tissues tested	Yes (2, 3, 14, 30)	Yes
Growth in chick embryo tissue culture	No (14)	No
Withstands 45 C for 30 min	Yes (27)	Yes
Inactivated at 55 C for 10 min	Yes (27)	Yes
Type A intranuclear inclusions	Yes (2, 3, 4, 14)	Yes
Pattern of intranuclear inclusions formation	Same (30)	Same
Inclusions present 48 hr postinfection	Yes (3)	Yes
Presence of infectious virus in cell-free culture fluid	Yes (2, 30)	Yes
In vivo		
Growth on CAM	No (14)	No
Visible effect on guinea pigs	No (27)	No
Natural infection asymptomatic in rabbit	Yes (6, 30)	Yes
Neutralizing antibodies present in "normal" rabbits	Yes (6, 30)	Yes

[a] Numbers in parentheses refer to literature citations.

culture during a brief asymptomatic acute illness? If HC is virus III, why has the latter gone undetected all these years? CPE of HC appears similar to normal PRK degeneration and could be easily overlooked. In addition, if our rabbit colony is typical, the prevalence of active renal infection with HC must be very low. No other recoveries of the agent have been made in preparing well over 100 lots of PRK in the 3 years since HC was first isolated. Interestingly, I have noted on quite a number of occasions an unrelated CPE which seemed to be consistent with the presence of rabbit vacuolating virus (13). CPE of HC does not resemble CPE of rabbit vacuolating virus in any way. None of the lots of PRK showing vacuolation were used in the preparation of HC or in experiments on HC.

The most important consideration raised by the isolation of this rabbit herpesvirus is that HC represents a largely ignored source of confusion for those using rabbits, rabbit antisera, or rabbit tissues in investigation of herpes group viruses. HC produces "herpes" type CPE in certain culture systems. HC produces typical herpes inclusions in stained tissue culture and in histological sections. HC appears identical to other herpesviruses in electronmicrographs. It is evident that identification of experimentally produced herpes group virus infection in rabbits by morphological criteria alone is not valid since the findings could represent unsuspected HC infection. As demonstrated in this study with antiserum against herpesvirus bovis, rabbit sera may contain unsuspected HC antibody, thereby invalidating serological or fluorescent antibody tests carried out on isolates or tissue from a rabbit.

The following methods for avoiding confusion when using rabbits or rabbit products in investigation of other herpes group viruses are suggested. (i) Hold all rabbit cell cultures for 3 weeks prior to use. With the medium described in this study, viable PRK monolayers may be kept for many weeks. (ii) Use cell cultures known to be

resistant to HC for isolation or passage of isolates obtained from rabbits or rabbit cell cultures. (iii) Check all isolates obtained in rabbit cell cultures for specificity by neutralization or other serological tests with an antiserum known to have no activity against HC. (iv) Do not use unchecked rabbit antiserum in fluorescent antibody staining of rabbit tissues, since unsuspected HC antibody may stain areas containing unsuspected HC antigen.

In contrast to other members of the herpes group which produce relatively rapid changes in infected tissues, HC is slow, which may make it a valuable experimental agent for studying certain aspects of herpesvirus biosynthesis and maturation.

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