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New Member of the Herpesvirus Group Isolated from Wild Cottontail Rabbits

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A viral agent has been isolated from naturally infected wild cottontail rabbits (*Sylvilagus floridanus*). The virus appears to have the general physical, chemical, and biological properties of the herpesvirus group. It differs significantly in host range and antigenic structure from the previously recognized herpesviruses and is proposed as a new member of this group of viruses. The name of *Herpesvirus sylvilagus* is suggested for the agent.

Within the last few years several new members have been added to the herpesvirus group. This has been brought about both by the discovery of new viruses in a variety of animals and by the reclassification of previously known agents which possess features characteristic of this group. The present report describes the isolation, from naturally infected cottontail rabbits, of a virus which fits the classification of the herpesvirus group and is sufficiently different from the established members to warrant its consideration as a new member of this group. A preliminary report of this agent has been previously presented (H. C. Hinze, Bacteriol. Proc., p. 149, 1968).

MATERIALS AND METHODS

Cell cultures. Primary cultures of cottontail and New Zealand white rabbit kidney cells were prepared by trypsinization by using established methods. The DRK line of diploid New Zealand white kidney cells (2) and a cloned subline (DRK-3) were maintained by weekly passage in 6-oz (ca. 180 ml) prescription bottles. Cultures of HeLa, Chang's conjunctiva, African grivet monkey kidney (Vero), human embryo lung, and human embryo skin were obtained through the courtesy of D. L. Walker of this Department. All other cell cultures were obtained from Microbiological Associates, Bethesda, Md., in the form of prepared monolayers and used without further passage.

All primary and serially cultured rabbit cells, as well as most other cells used in this work, were grown in medium 199 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 0.25% lactalbumin hydrolysate and 10% heated calf serum. Established monolayers were maintained in a similar medium in which the concentration of calf serum was reduced to 3%. The few cell types not adapted to the above media were grown in Eagle's basal medium containing 10% calf serum. All media contained 100 units of penicillin per ml, 100 μg of streptomycin per ml, and sufficient sodium bicarbonate to maintain a *p*H of 7.4 to 7.8.

Virus. Stock preparations of the newly isolated cottontail herpesvirus (CHV-1) were grown in cloned rabbit kidney cells of the DRK-3 line. Maximum yield of infectious virus was obtained by harvesting infected cultures in medium containing 30% glycerine and storing at −65 C. Attempts to purify virus preparations by ultrasonic treatment and centrifugation to remove cell debris resulted in a drop in virus titer of 1 to 2 logs. Such procedures were, therefore, not routinely done.

Antiserum. Antiserum was prepared against CHV-1 both by infection of adult cottontail rabbits and by repeated inoculation of concentrated virus preparations into New Zealand white rabbits. Serum was collected 12 to 14 weeks after infection of the cottontail rabbits and 1 to 2 weeks after the final injection of virus in New Zealand whites. Hyperimmune sera of known potency against *Herpesvirus hominis*, *H. cuniculi*, *H. saimiri*, *H. suis*, and *H. tamarinus* were obtained through the courtesy of L. V. Melendez, Harvard Primate Research Center, Southboro, Mass.; H. E. Hopps, National Institutes of Health, Bethesda, Md.; and F. W. Deinhardt, Presbyterian-St. Luke's Hospital, Chicago, Ill.

Virus assay. Plaque assays were carried out on monolayers of DRK-3 cells grown in 30-ml plastic tissue culture flasks (Falcon Plastic, Div. of BioQuest, Los Angeles, Calif.). Cultures inoculated with 0.5-ml amounts of appropriate virus dilutions were incubated at 37 C for 1 hr to permit virus adsorption. After the adsorption period, the monolayers were covered with a nutrient agar overlay composed of Hanks balanced salt solution (BSS, without phenol red) containing 0.5% lactalbumin hydrolysate, 0.1% bovine albumin, 0.1% yeast extract, 6% horse serum, and 0.9% Difco purified agar. After incubation at 35 C for 10 to 14 days, the cell sheets were stained through the overlay by addition of 1 ml of 1:1,000 neutral red dissolved in distilled water. Plaques (average diameter 2 to 3 mm) were easily countable against a white background.

Serum neutralization. Serum neutralization tests were performed with the plaque reduction method. Twofold serum dilutions were mixed with equal

volumes of virus diluted to contain 100 plaque-forming units (PFU) per 0.5 ml and incubated for 1 hr at room temperature. The virus-serum mixtures, together with appropriate controls, were then assayed for residual infectious virus by the methods described above. A 50% reduction in plaque number was taken as the neutralization end point.

Electron microscopy. Infected DRK-3 cells were prepared for examination by the method of Parsons (8). Cell fragments were floated on the surface of a 2% solution of potassium phosphotungstate (*p*H 6.0) and picked up on copper grids covered with a carbon-coated Formvar film. The specimens were examined in an RCA model 3-G electron microscope. Photographs were taken at an initial magnification of 33,500.

RESULTS

Virus isolations. The original isolation of this virus was made from primary cell cultures prepared from the pooled kidney tissue of three apparently healthy weanling cottontail rabbits (*Sylvilagus floridanus*) trapped in southern Wisconsin. The cultures were incubated at 37 C with routine medium changes and appeared to grow in a normal manner. Focal areas of cell destruction were observed approximately 2 weeks after initiation of the cultures. These areas enlarged slowly to involve most cells in the cultures during the next 7 days. Propagation of the agent was at first carried out by inoculation of cells and fluid from the initially infected cultures into monolayers of normal cottontail rabbit kidney cells. After four serial passages in cottontail cells, growth was obtained both in primary and serially cultured (DRK-3) kidney cells of New Zealand white rabbits. At the present time, the virus has been carried through a total of 20 tissue culture passages.

In addition to the initial isolate (CHV-1), the same agent has been isolated on five subsequent occasions from cottontail rabbits trapped in different areas of central and southern Wisconsin. These isolations were made on DRK-3 monolayers by inoculating 0.5 ml of fresh defibrinated blood from the infected animals together with 4.5 ml of nutrient medium. The inocula were left on the cells for 2 days after which the cultures were washed to remove red cells, refed with maintenance medium, and incubated at 37 C for 14 to 21 days. In all instances, specific viral cytopathic effects appeared in 10 to 15 days after inoculation.

Cultural characteristics. Growth of CHV-1 in both cottontail and domestic rabbit kidney cells was initially slow with noticeable cytopathic effects appearing after 10 to 15 days of incubation. With repeated passage in tissue culture, this time interval has been reduced and specific cytopathic effects are now seen regularly within 3 to 6 days after inoculation of high concentrations of virus. The type of cell destruction produced is similar in both cottontail and domestic rabbit cells. Infected monolayers first show focal areas of round, distorted cells (Fig. 1A). Within 1 to 2 days, these areas develop large multinucleated syncytial masses often containing 50 or more nuclei (Fig. 1B). Eosin and hematoxylin stains of such cultures show typical type A intranuclear inclusions in all infected cells. Infected cultures maintained at 37 C with frequent medium changes show a progressive and complete cell destruction over a period of 5 to 7 days. The yield of virus obtained from cultures exhibiting almost complete cell involvement averages 5×10^5 PFU per ml and seldom exceeds a titer of 10^6 PFU per ml.

Although growth of this agent in rabbit cells has been readily accomplished, no indication of virus replication has been obtained in primary or serially cultured cells from a number of other animal species. Negative results have been obtained with the following cell types: human embryo lung, human embryo skin and muscle, WI-38, HeLa, Chang's conjunctiva, human amnion (FL), green monkey kidney (Vero), primary rhesus monkey kidney, primary hamster kidney, BHK-21, primary mouse embryo, and primary chick embryo.

Virus host range in animals. Inoculation of CHV-1 by the intradermal, subcutaneous, or intraperitoneal route into cottontail rabbits consistently produces a chronic infection in which virus persists in the blood, presumably in the leukocytes, for the remainder of the life of the animal. The effect of this virus in producing a lymphoproliferative disease in these animals is described elsewhere (H. C. Hinze, Bacteriol. Proc., p. 157, 1969).

Repeated attempts have been made to produce a detectable infection with CHV-1 in domestic New Zealand white rabbits (*Oryctolagus cuniculus*). In the first attempt, 6 weanling and 10 adult New Zealand whites were inoculated intraperitoneally with 10^5 PFU of virus. At 1, 3, 7, 14, 21, and 42 days, the animals were examined for the presence of virus in the blood, since this method has been found to be most accurate in detecting infection in susceptible cottontail rabbits. In addition, two animals were killed at 7 and 14 days, and the liver, spleen, lymph nodes, and kidneys were removed. Portions of each organ were ground in BSS and inoculated into DRK cultures for detection of virus. The remaining animals were sacrificed at 21 and 42 days, and the organs were examined for the presence of

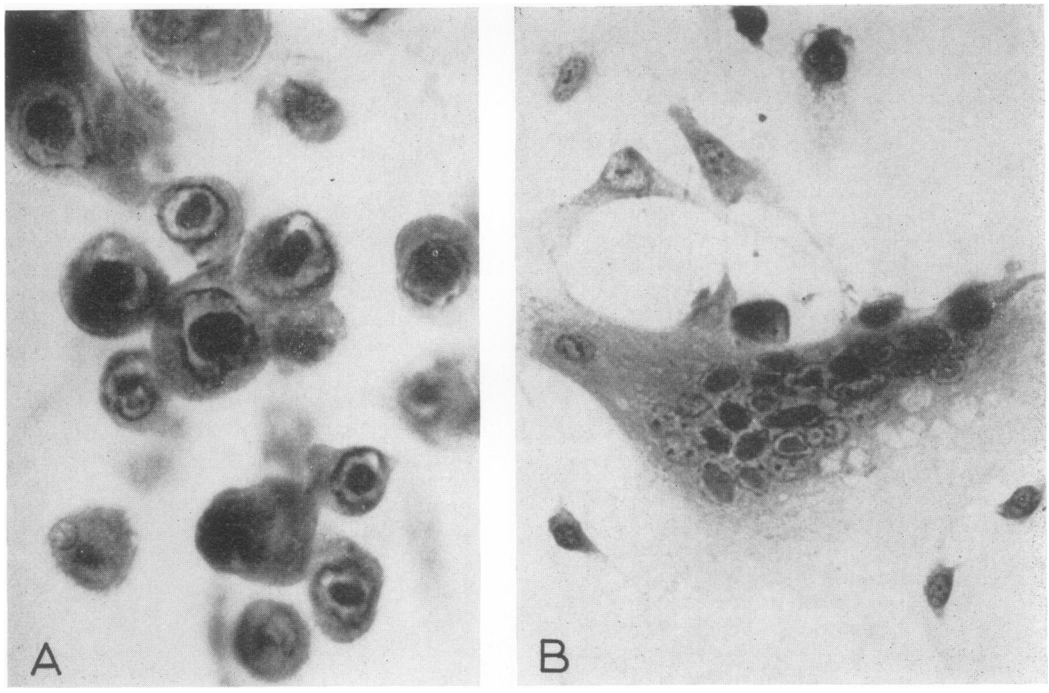

FIG. 1. *(A) DRK cells fixed 3 days after infection with CHV-1. Focal areas of cell destruction are made up of round, distorted cells containing characteristic type A intranuclear inclusions. Hematoxylin and eosin stain. × 970. (B) DRK cells fixed 5 days after infection with CHV-1. Photograph shows developing syncytial cell with many inclusion-containing nuclei. Hematoxylin and eosin stain. × 300.*

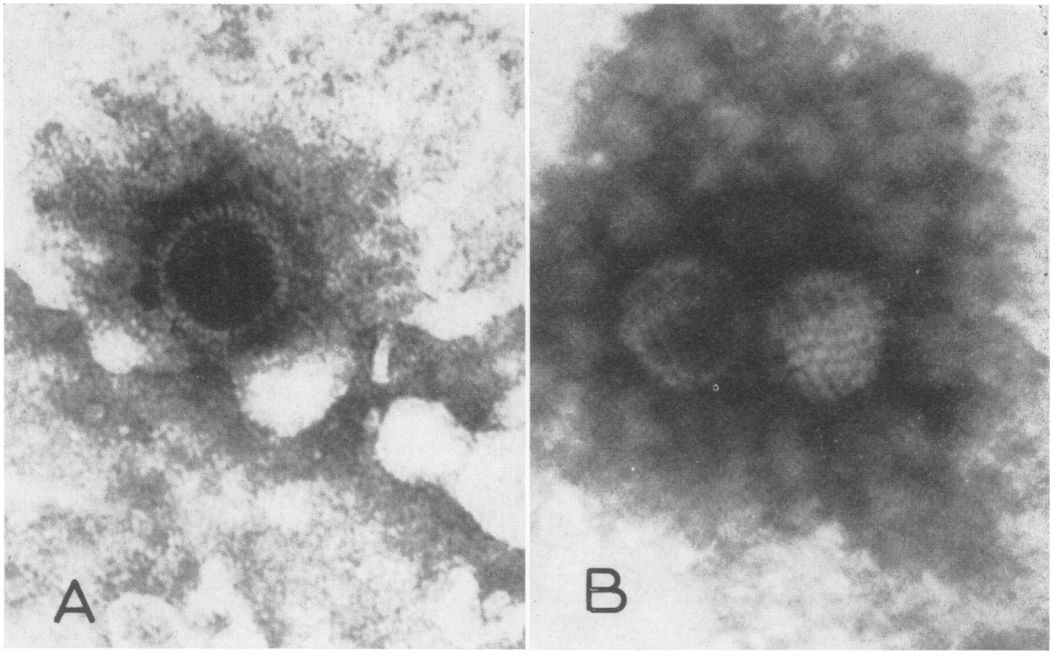

FIG. 2. *Electron micrographs of negatively stained CHV-1 particles in fragments of infected DRK cells. (A) "Empty" capsid showing hollow capsomeres. × 390,000. (B) Particles containing a full or partial complement of nucleic acid. × 390,000.*

pathological changes in stained tissue sections. No signs of clinical illness were observed in any of the animals at any time. No virus was recoverable from the blood or tissues at any interval tested, nor were pathological changes related to infection seen in the tissue sections. Examination of the sera from those animals sacrificed at 21 and 42 days showed no neutralizing antibody present.

Additional attempts to infect New Zealand white rabbits by the intravenous, intradermal, subcutaneous, intramuscular, and intracerebral routes were without success. Inoculation of 10^5 PFU of virus onto the scarified cornea also produced no lesions. Four serial blind passages of CHV-1 by the intratesticular route through adult male New Zealand white rabbits at intervals of 4 to 5 days failed to produce detectable lesions or recoverable virus by tissue culture inoculation.

Other animals which have been found resistant to infection with this agent include newborn and adult albino Swiss mice, newborn and adult Syrian hamsters, and adult guinea pigs. Inoculation of CHV-1 onto the chorioallantoic membrane of embryonated eggs failed to produce lesions or recoverable virus when examined at 2, 4, and 6 days after inoculation.

Nucleic acid type. Evidence that this virus contains deoxyribonucleic acid (DNA) as its nucleic acid was obtained by the differential staining of infected cells with acridine-orange before and after treatment with ribonuclease and deoxyribonuclease by the method described by McAllister et al. (5). Such treatment demonstrated the presence of green staining (DNA-containing) intranuclear inclusion bodies in cells showing viral cytopathic effects. Treatment with deoxyribonuclease, but not with ribonuclease, completely destroyed the staining characteristic of the virus inclusions.

Additional evidence of the DNA content of this virus was obtained by the use of DNA inhibitors. Groups of DRK-3 cultures were inoculated with 10^5 PFU of CHV-1 and incubated at 37 C for 2 hr to permit virus adsorption. The cultures were then washed to remove free virus and refed with medium containing either 10^{-5} M 5-bromodeoxyuridine, 10^{-5} M 5-iododeoxyuridine, or medium without inhibitor. After 4 days of incubation at 37 C, the cultures were assayed for infectious virus. Cultures containing either inhibitor showed no virus present in the cultures, whereas the controls, without inhibitor, contained approximately 5×10^4 PFU of virus.

Heat stability. Heat inactivation of CHV-1 was determined by submerging sealed glass ampoules containing 1-ml samples of virus in a water bath at 56 C. At intervals of 5 min, ampoules were removed, chilled, and assayed for residual infectious virus. The initial titer of 10^6 PFU per ml was reduced to 4×10^2 PFU per ml at 5 min. Samples removed at 10 min or longer showed no infectious virus remaining.

Ether stability. Destruction of infectivity by ether treatment was demonstrated by mixing 5-ml samples of virus containing 10^6 PFU per ml with sufficient anhydrous diethylether to give a final ether concentration of 20% or with an equivalent volume of BSS as a control. The mixtures were immediately chilled and held at 4 C for 18 hr. After the 18-hr period, the ether was removed and residual virus was determined by plaque titration. No infectious virus was detected in the ether-treated samples, whereas controls showed a decrease of approximately 1 log.

Acid stability. The inactivation of CHV-1 at *p*H 3.0 was carried out by the method of Ketler et al. (3). Stock virus was diluted 1:10 in tissue culture medium adjusted to *p*H 3.0 or 7.2. After 3 hr at room temperature, the residual infectious virus in each sample was determined by titration on DRK-3 monolayers. The virus titer before treatment was 2.4×10^5 PFU per ml. The sample held at *p*H 3.0 for 3 hr had a residual virus titer of less than 10^1 PFU per ml, whereas that held for the same time at *p*H 7.2 had a titer of 1.2×10^5 PFU per ml.

Size. An estimation of the size of the infectious agent was obtained by filtration through membrane filters of graded pore size. Although ultrasonic treatment of stock virus preparations generally reduced the infectious titer by 1 to 2 logs, the remaining virus passed readily through filters of 200 nm and larger but was withheld by filters of 100 nm and smaller.

Antigenic relationship to other herpesviruses. The antigenic relationship of CHV-1 to other members of the herpesvirus group was examined by plaque reduction neutralization. Hyperimmune sera prepared against *H. hominis*, *H. suis*, *H. cuniculi*, *H. tamarinus*, and *H. saimiri* showed no neutralizing activity when tested with the newly isolated agent. CHV-1 antiserum having a neutralizing titer of 1:128 when tested against the homologous agent gave no neutralization of *H. hominis* and *H. suis*.

Electron microscopy. A preliminary study of the morphology of the CHV-1 virion was carried out to determine its similarity to other herpesviruses. DRK-3 cells were harvested 6 and 9 days after infection with CHV-1 virus and prepared for electron microscopic examination by negative staining with potassium phosphotungstate. Both "empty" and "full" capsids were observed in most preparations (Fig. 2A and B). The capsids measured approximately 90 nm in diameter and

consisted of an icosahedral structure composed of regularly arranged hollow, elongated capsomeres. No distinct envelop was seen. The number of capsomeres, 162, estimated from the photographs is consistent with other members of the herpesvirus group.

DISCUSSION

The agent described in this report (CHV-1) appears to fulfill the taxonomic criteria of the herpesvirus group by all characteristics thus far examined. It is a DNA virus, ether- and acid-labile, readily destroyed by heat, and of the same general size as other members of this group. In addition, the agent induces formation of type A intranuclear inclusions identical to those formed by other herpesviruses. CHV-1 appears to be antigenically distinct from the other herpesviruses with which it has been compared. Although electron microscopic examination of CHV-1 has not yet been completed, preliminary studies indicate the virus has a physical structure consistent with that of the herpesvirus group.

Ley and Burger (4), studying the multiplication and release of CHV-1 in tissue culture, have found the virus to be primarily a cell-bound agent with less than 1% of the replicating virus released into the superatnant medium. From their data, they suggest that this virus belongs in herpesvirus subgroup B. Results in this laboratory are in agreement with that finding.

To establish this agent as a new member of the herpesvirus group, it has been necessary to rule out the possibility that CHV-1 is similar, or identical, to the virus III (*H. cuniculi*) known to produce a latent natural infection in rabbits of the genus *Oryctologus*. Although the original isolates of virus III are no longer available, early work by Rivers and Tillett (9) and Andrews et al. (1, 6), clearly describes the ability of that virus to induce lesions in the skin, cornea, and testicle of "laboratory" rabbits (presumably *Oryctologus*) after rapid serial passage through such animals. Repeated attempts to produce similar results with CHV-1 indicate that New Zealand white rabbits are resistant to infection with this virus. This resistance is most probably not an immunity resulting from previous natural infection, since these animals lack neutralizing antibody in their sera, but rather appears to be a genetic insusceptibility to infection by this virus. Additional evidence distinguishing CHV-1 from virus III is the failure of hyperimmune serum prepared against the recently isolated *H. cuniculi* (7), presumed to be virus III, to neutralize CHV-1.

It appears from the results obtained in this study that the agent isolated from naturally infected cottontail rabbits is unrelated to other known members of the herpesvirus group and most probably represents a new member of that group of viruses. In keeping with the recent trend in naming viruses of this group, the name of *Herpesvirus sylvilagus* is suggested for this agent.

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