Herpes Virus Aotus: A Latent Herpesvirus from Owl Monkeys (*Aotus trivirgatus*) Isolation and Characterization[1] (36002)

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(Introduced by B. F. Trum)

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The use of new-world monkeys in biomedical research has increased during recent years. The greater use of these species has led to the isolation of many new viral agents (1). The most rewarding finding in these studies has been the isolation of an oncogenic agent, herpesvirus saimiri (2). To characterize H. saimiri, owl monkey kidney cell cultures were employed routinely in this laboratory; and, by so doing, we were able to isolate several latent agents from these cell cultures. This paper describes the isolation and full characterization of a new herpesvirus among these owl monkey kidney isolates. After the isolation and preliminary identification, we suggested that this herpesvirus be called herpesvirus aotus (3).

Cell cultures. Primary owl monkey kidney cell cultures (OMK) were prepared from kidneys removed from apparently healthy owl monkeys (*Aotus trivirgatus*). These were prepared by trypsinization according to methods previously described (4, 5). Squirrel monkey kidney cell cultures (SMK), Cebus monkey kidney (CMK) cell cultures, and rabbit kidney (RK) cell cultures were prepared in the same manner as described above.

Vero (African green monkey kidney) cell line originally received from Dr. J. S. Rhim, National Cancer Institute, Bethesda, MD and maintained in our laboratory; whole human embryo and human embryo skin and muscle purchased as a primary from Microbiological Associates and maintained in our laboratory; human embryonic lung cells originally received from Professor J. F. Enders, Harvard Medical School, Boston, MA and maintained in our laboratory were also employed in this study. BSC-1 cells were kindly provided by Dr. Robert Rustigian, VA Hospital, Brockton, MA.

Owl monkey cornea cell cultures (OMC) were also employed. The cornea from both eyes was removed and washed in calcium magnesium-free phosphate buffer. The washed cornea was finely minced with scissors. The minced tissue fragments were washed again and treated in one of two ways:

1. Trypsinization by the conventional method and seeding the cell suspension in 60 mm dishes.

2. Placing tiny fragments of minced tissue in several places on a 18 mm coverslip placed in a 35 mm Falcon plastic dish. This coverslip was overlaid with another 18 mm coverslip so as to make a sandwich. Medium was added by capillary action with a sterile pasteur pipette. The dishes were placed in a CO_2 incubator maintained at 37° and were refed once or twice during the course of the day. On the next day about 2 ml of medium were added. Subsequent media changes were done, once or twice a week, depending on the acidity of the growth medium. Cell growth developed within 1 to 2 weeks. The growth was allowed to spread on the entire surface of the coverslip and into the plastic portion of the dish. At this point the coverslips were separated by means of forceps and the cells were trypsinized by the conventional method. The cell suspension was diluted 1:100 in growth medium and dispensed in 30 or 250 ml Falcon plastic flasks. In the initial stages, addition of 20% fetal calf serum in the media stimulated better growth.

Goat synovial bursa and capsule cell cul-

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tures. The synovial bursa from the knee joint of a young goat was removed under sterile conditions. The bursal part and the capsular portion were separated and each minced with a pair of scissors and treated in the same way as described for OMC. The bursal cells had more epithelial-like cells and less fibroblastic-type cells. The capsular portion contained more fibroblastic-type cells.

Hamster heart cell culture was prepared by pooling the hearts of eight 1-day-old Syrian hamsters which were treated in the same way as for owl monkey kidney cell cultures.

Egg inoculation. White leghorn eggs were obtained from a commercial supplier. Chorioallantoic membrane inoculation of 10-day-old embryonated eggs was performed according to standard techniques (6).

Virus. Primary OMK cell culture fluid was harvested when cytopathic effect (CPE) was maximal. Aliquots of this cell virus suspension were stored at —186 and at —86° in glass ampules and screw capped vials. Experiments were carried out employing any one of the first eight passages. This isolate 588 was termed H. aotus.

Other viruses employed besides H. aotus, OMKI 372 and 68-69 were standard viruses maintained in the herpesvirus repository in our laboratory. OMKI 372 and 68-69 were isolated by one of us (MDD and LVM) respectively, and preliminary information on these agents has been reported (7).

Virus titration. Virus titrations were carried out in 35 mm Falcon plastic dishes containing a coverslip (18 mm). These cultures were incubated with 0.1 ml of serial 10-fold viral dilutions and inoculated at 37° in a CO_2 incubator. End points were determined within 14 to 40 days of incubation by the appearance and progression of CPE. The 50% infective dose ($TCID_{50}$) was determined by the method of Reed and Muench (8).

Plaque titration. OMK and SMK cells were employed for plaque titration under an agar or methylcellulose overlay as previously described (9, 10).

Neutralization. OMK cell cultures were employed for serum neutralization test. A 1:5 serum dilution was tested against varying tenfold virus dilutions according to standard techniques (6). The virus–serum mixture was held for 2 hr at 4°.

Antiserum preparation. Four-month-old New Zealand albino rabbits were employed. The virus employed for inoculation of rabbits had a titer of $10^{4.5}$/ infected OMK cultures were subjected to ultrasonic treatment (3 cycles of 10 sec duration in 30 ml volumes) in a Biosonik sonifier (Bronwill Scientific, Rochester, New York), after which the viral fluid was centrifuged for 10 min at 2000 rpm; and the supernatant fluid was used as inoculum. Two rabbits were initially inoculated, each with 1 ml of virus subcutaneously (sc); and 2 each with 1 ml intravenously (iv). At 10-day intervals these animals were reinoculated employing the same routes of inoculation. Prior to each inoculation the rabbits were bled for antibody (AB) detection. After 2 months as no AB were detected, each of the rabbits was inoculated intraperitoneally (ip) with virus mixed in complete Freund's adjuvant (FA) (1.5 ml of virus + 1.5 ml FA). Subsequently these animals were inoculated every other week with fresh virus iv and sc for a total period of 6 months and once by corneal scarification (0.05 ml dilution virus in the left eye).

Antiserum preparation in goats. Two goats were inoculated intradermally with 1 ml of virus at multiple sites. These goats were bled prior to each inoculation and they were bled on days 7, 14, 22, 28, 35, and 42 postinoculation. Two booster inoculations each of 1 ml were administered on days 22 and 35.

Other antisera. Herpesvirus T; Herpesvirus hominis; infectious bovine rhinotraecheitis (IBR), Herpesvirus saimiri, Herpesvirus B were standard antisera maintained in our laboratories. Their preparation has been described previously (11). Antisera to marmoset kidney isolate, Herpesvirus saguinus (3) were prepared in goats similarly to Herpesvirus aotus. OMKI 372-70 (another owl monkey herpesvirus), antisera were prepared in rabbits by inoculating 2 ml iv initially followed by 1 ml weekly for 4 weeks. OMKI 68-69 antisera (another herpesvirus from owl monkeys) were prepared in goats in the same way as H. aotus.

Staining procedures. Hematoxylin and eo-

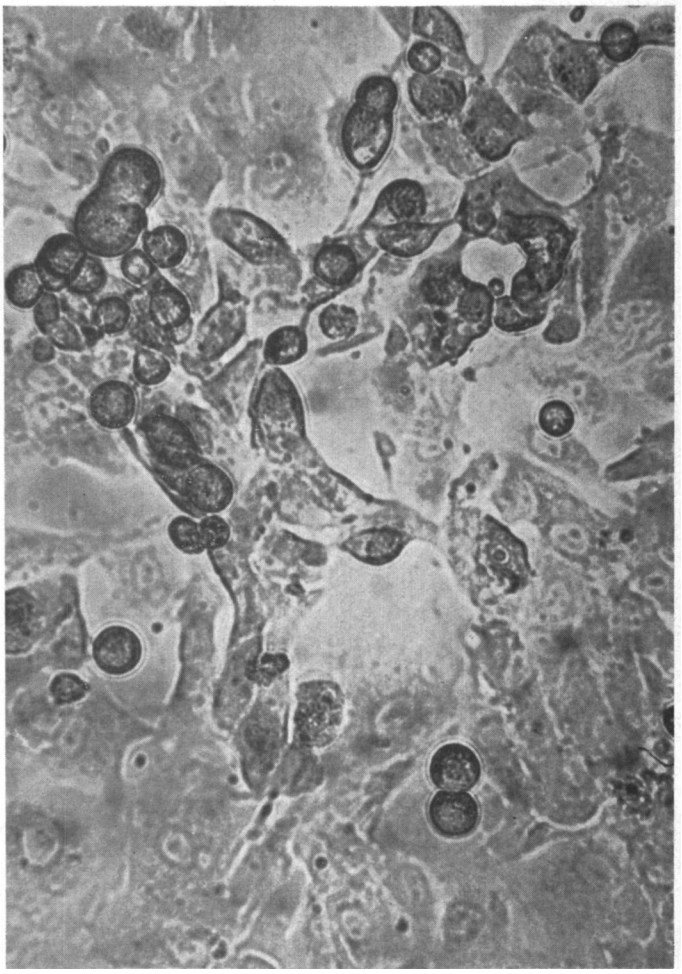

FIG. 1. Early cytopathic effect of H. aotus in owl monkey kidney cell cultures, showing rounded refractible cells in clumps; unstained, ×220.

sin staining of monolayer cultures was done after fixation in Bouin's solution. The method of acridine orange staining (AO) has been described (12). Fluorescent antibody staining–immunofluorescence studies were carried out by the indirect method, similar to that described for H. saimiri (13).

Electron microscopy. A monolayer of owl monkey kidney cells inoculated with H. aotus was rinsed with several changes of Sorensen's phosphate buffer (pH. 7.4); and then fixed for 2 hr at room temperature with 1% glutaraldehyde in phosphate buffer. After fixation, the cells were again rinsed with buffer and postfixed with 2% osmium tetroxide in phosphate buffer at 4° for 1 hr. Following osmification, the cells were scraped from the flask, centrifuged into a pellet, and the pellet was dehydrated through a graded series of ethanol. The pelleted cells were embedded in Epon 812 and sectioned with a diamond knife using a Porter-Blum ultramicrotome. Thin sections were mounted on clean 200 mesh copper grids and stained with uranyl acetate and lead citrate. The sections were examined with an R.C.A.-EMU3-H electron microscope employing 50 kV accelerating voltage.

Results. Owl monkey 588-68 kidney had more than 50% growth confluency in 4 days. In 10 days, the cell culture attained full confluency. Some cultures were transferred

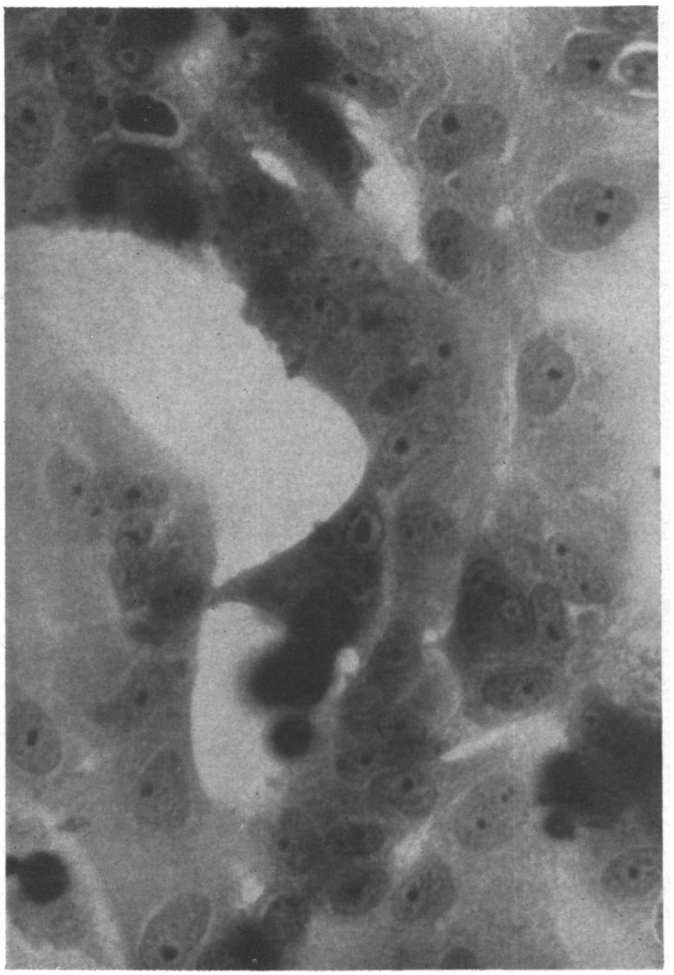

FIG. 2. Typical herpes type A inclusions as seen in the original seeding S-34E 25 days after the kidney cultures were initiated; H and E staining, ×400.

and others were kept for long-term observation. The medium was changed twice weekly.

On day 23 after the initiation of the primary, cellular alteration in the form of small clumps of rounded cells was noticed. In the next 4 to 5 days, these areas increased and focal areas of CPE became discernible. These consisted of swollen refractile cells, rounded and gathered in clumps (Fig. 1). There were many cells with pyknotic nuclei and others with type A intranuclear inclusions similar to those described for herpesviruses (Fig. 2) and very few polykaryocytes when the stained preparations were observed.

Fluid from flasks and dishes which showed maximum CPE was collected as viral isolate S-34E. The remaining cultures were left for development of CPE. Those that failed to show CPE were transferred (only one flask). This flask was kept for as long as 122 days and when no CPE was seen these cultures were transferred up to a total of 16 transfers during a period of 7 months and during this period no CPE appeared. All other dishes and flasks including those that were transferred developed CPE within 23 to 30 days.

Cytopathogenicity. Herpesvirus aotus grew best in OMK or OMC cell cultures. Inoculation of fresh undiluted virus having a titer of 10^6/ml produced cellular alteration within 48 hr. This cell alteration progressively increased to maximum CPE within 1 week.

Inoculation of frozen virus delayed the appearance of CPE to beyond 5 days with maximum cell destruction to beyond 14 days. In OMK cultures, the virus produced small, hardly discernible polykaryocytes composed of 5 to 8 nuclei. The formation of polykaryocytes was not seen in any other susceptible cell cultures. The virus reached a titer of 10^6/ml in OMK or OMC when inoculated with fresh virus at the end of 3 weeks. With frozen virus, the titer dropped to $10^{4.5}$/ml during a 3-week observation period. Other cell cultures in which the virus grew, although poorly, were CMK, BSC-1, Vero, HEL, WHE, human embryo skin and muscle; and no CPE were seen in RK, chimpanzee lung, LLC-MK_4, hamster heart, and goat cells.

Plaque formation. Herpesvirus aotus failed to form plaques under agar overlay when tested with 0.5 and 1% Difco Bacto agar. Under 1% methylcellulose, small plaque-like areas were seen, but they were difficult to enumerate.

Pathogenicity. CAM of embryonated eggs failed to support the growth of H. aotus and no pocks developed. Repeated inoculation of rabbits for a period of 6 months produced no detectable disease. Only very low levels of neutralizing antibodies were detected. The titer (neutralization index) ranged from 0.5 to 1.0 log. In goats, too, the virus had no effect; however, the antibody level in goats reached a slightly higher titer (1.5 log) in 21 days. Unlike most other herpesviruses, H. aotus appeared to be a poor antigen in both rabbits and goats. Corneal scarification with instillation of virus midway during immunization of rabbits did not cause any visible effect as previously described for Herpes T. (14)

Physical and chemical characteristics. The infectivity of the virus was destroyed by ether and heat (30 min at 56°). Treatment of virus with 5-bromodeoxyuridine also inhibited infectivity. The virus passed 220 nm filter but not 100 nm filters. AO staining of infected coverslip cultures gave intense DNA-type staining (yellow-green) within the nucleus corresponding to the inclusion seen under HE preparations. This was not seen in noninfected cell cultures.

Fluorescent microscope. Fluorescent antibody (FA) staining by the indirect test showed fluorescence of the cytoplasm and nucleus of infected cultures. Antisera to H. saimiri, OMKI 68-69, spider monkey herpesvirus (Lennette-Hull), Herpesvirus T. and H. simplex failed to cross react with H. aotus antigen. The antiserum to H. aotus had only a low fluorescent antibody titer, *i.e.*, 1:10. The present FA data supports neutralization data which showed H. aotus was unrelated to the above-mentioned herpesviruses.

The homologus antisera used for FA studies also had a low neutralizing titer (*i.e.*, NI of 1.5 log). We have so far not been able to obtain antisera with higher neutralizing titers. Further attempts will be made to obtain higher titered antisera to extend these studies.

EM appearance. Ultrastructure. The most striking ultrastructural changes occurred in the nuclei of infected cells. Nuclei became very elongated and developed prominent indentions and constrictions resulting in bizarre shapes. The chromatin was generally disposed at the periphery against the nuclear membrane. Some cells contained large densely staining nucleoli, which were not obviously displaced. Infected nuclei developed large, somewhat ill-defined reticulated masses of moderately dense granular material in their centers. This granular material was not as dense as chromatin or the nucleolus and was arranged in irregular anastomosing cords separated by a matrix of finely granular, less dense material (Fig. 3). Viral capsids were commonly associated with these granular inclusions.

The viral capsids were of three general types, all roughly round and 106.0 nm in diameter (Fig. 4). There were "empty" capsids, capsids with double shelled cores, and capsids with dense cores. In the cells examined, the capsids with double shelled cores were most numerous, followed by those with dense cores and lastly by those which appeared empty.

Enveloped capsids were found budding either through the inner leaflet of the nuclear membrane or into cytoplasmic vacuoles.

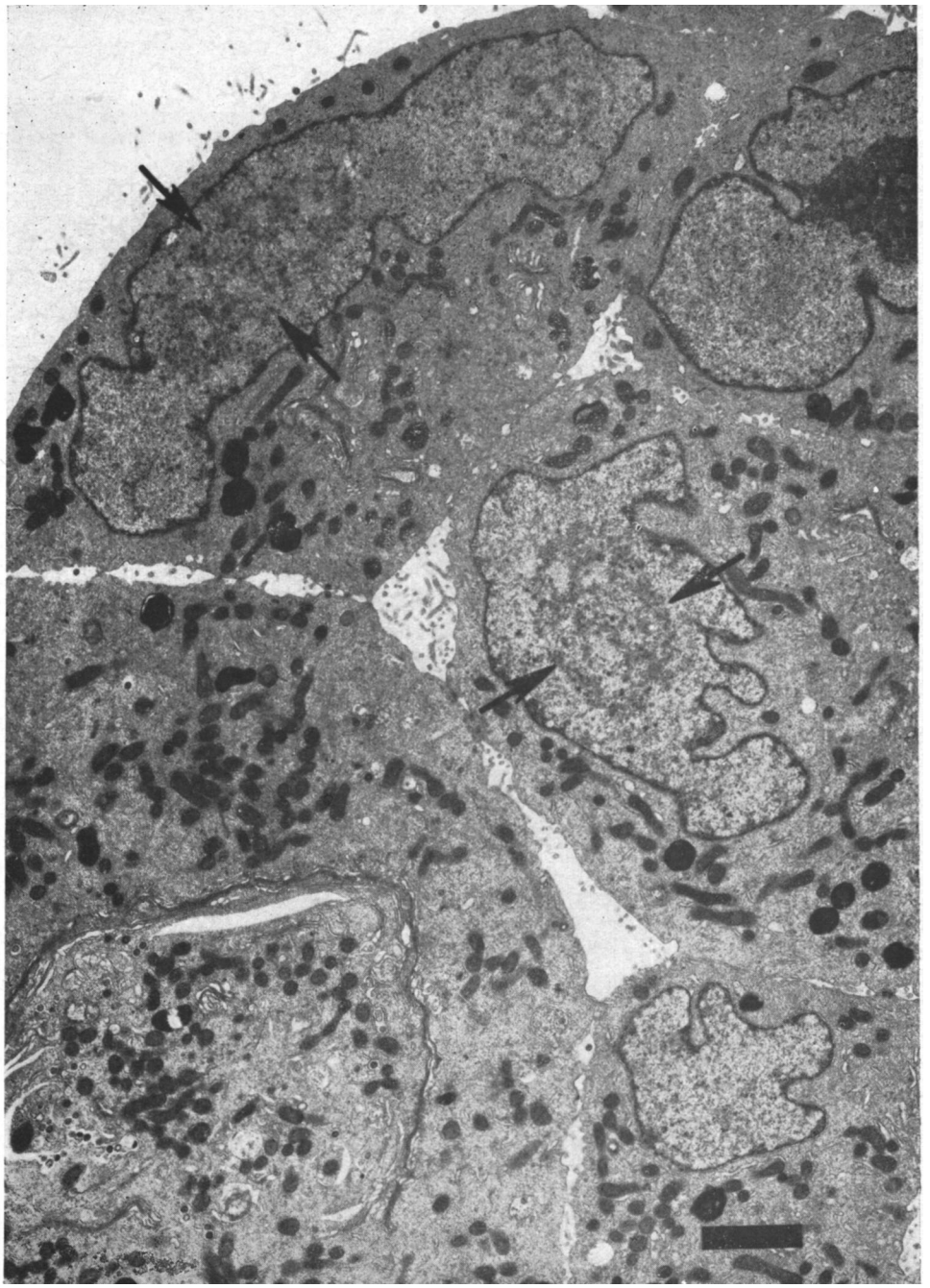

FIG. 3. The nuclei of 2 of the 5 OMK cells in this photograph contain moderately dense inclusion bodies (arrows). The nucleus in the cell on the upper right contains a dense nucleolus. Enveloped capsids are present within cytoplasmic vacuoles in the cell at the lower left. Bar = 2.0 μ, $\times$6950.

These particles were approximately 150 nm in diameter (Fig. 5).

The morphologic features of this agent are consistent with those of the herpesvirus group.

Histopathology. Gross and histopathological examination of the inoculated rabbits did not reveal any lesion indicative of a viral infection.

Serology. The infectivity of H. aotus was

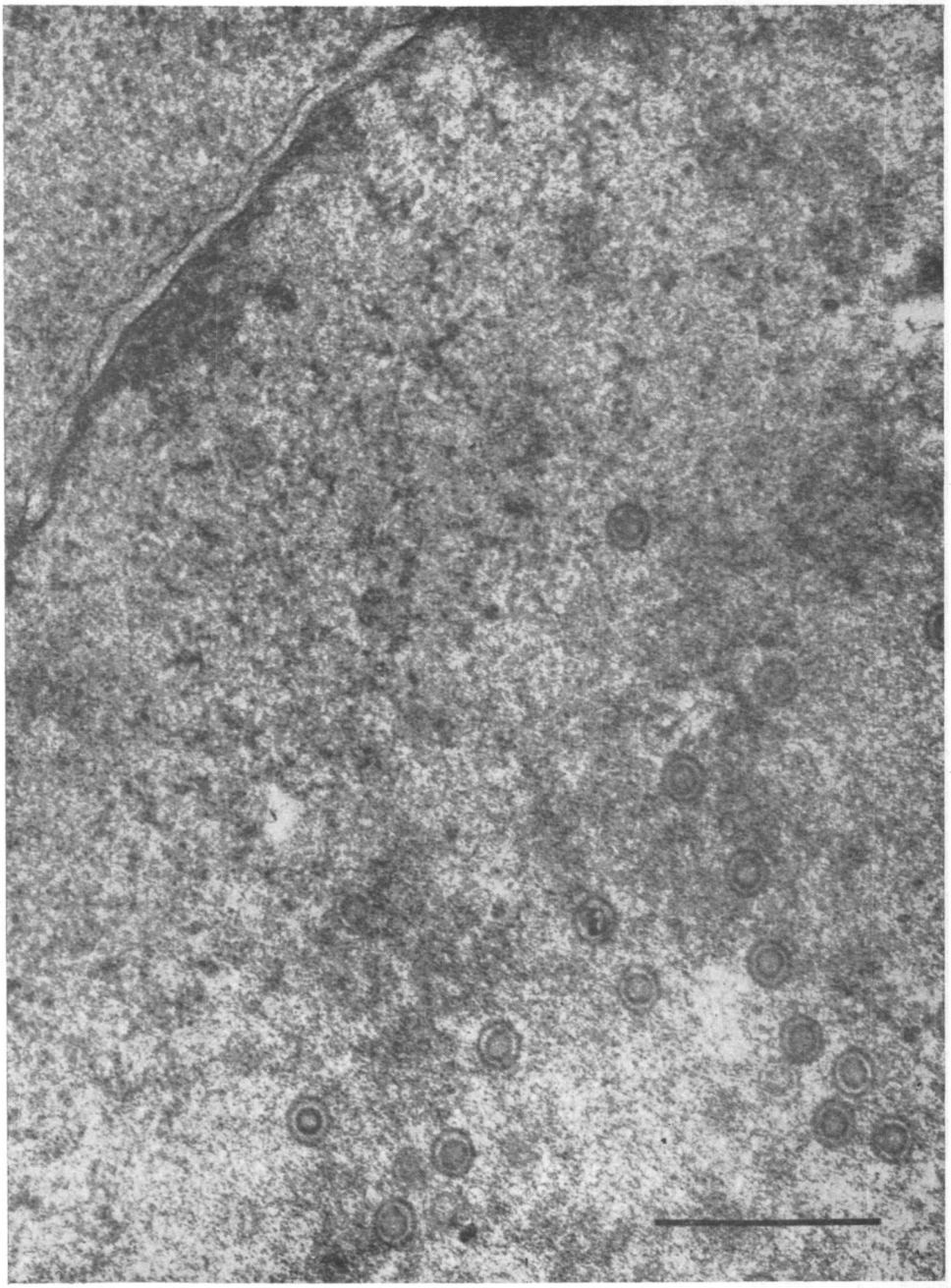

FIG. 4. A portion of a nucleus of an infected cell in which there are several capsids containing cores of the "double-shelled" variety. Bar = 0.5 μ, ×60,000.

not neutralized by Herpesvirus T, H. simplex, Herpesvirus B, H. saimiri, IBR, OMKI 372-70, or OMKI 68-69 antisera (Table I). Herpesvirus aotus antisera also failed to neutralize the infectivity of any of the above mentioned viruses. Testing of random sam-

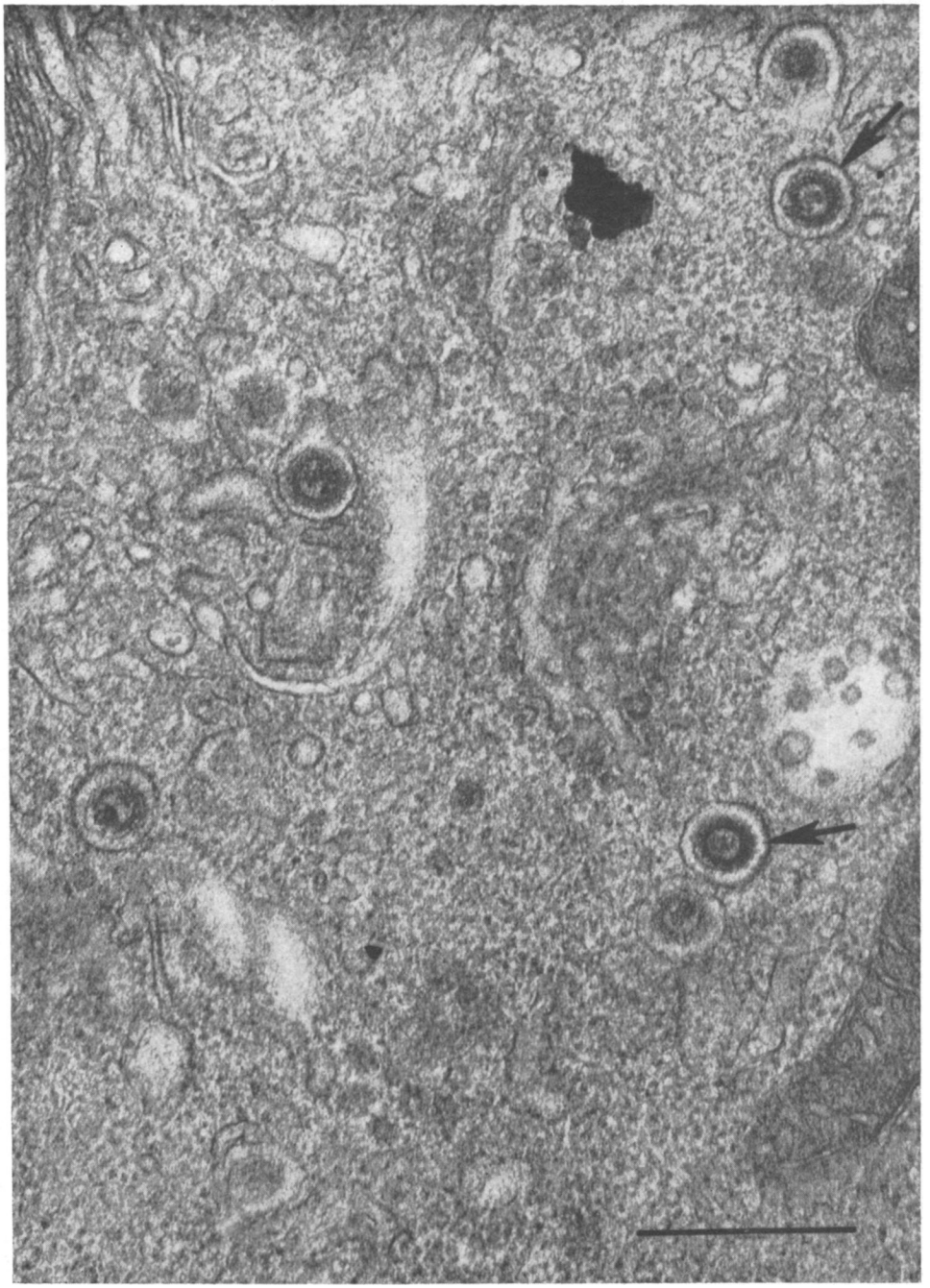

FIG. 5. Several enveloped capsids are present in cytoplasmic vacuoles. Some of them appear to have acquired their envelope by budding into these vacuoles. Note the thickened, envelope-like portions of some vacuolar membranes (arrows). Bar = 0.5 μ ×60,000.

TABLE I. Reciprocal Neutralization Indices of H. aotus and Other Herpes Viruses.

Viruses	Antisera							
	HS	HB	HT	HVS	HA	IBR	372	68
Herpes virus simplex	3.5[a]	0	0	0	0	0	0	0
B	0	3.5	0	0	0	0	0	0
T	0	0	3.5	0	0	0	0	0
saimiri	0	0	0	3.0	0	0	0	0
aotus	0	0	0	0	1.5	0	0	0
IBR[b]	0	0	0	0	0	3.5	0	0
OMKI 372-70	0	0	0	0	0	0	2.5	0
OMKI 68-69	0	0	0	0	0	0	0	1.5

[a] Expressed as neutralization indices.
[b] IBR infectious bovine rhinotracheitis virus.

ples of owl monkey sera has shown AB titers ranging from 0 to 3.0 log against H. aotus (Table II). Of 34 owl monkey sera tested, 30 showed the presence of varying levels of neutralizing antibodies.

Discussion. Isolate 588-68 (H. aotus) obtained from uninoculated primary OMK cell cultures was found to possess features attributed to the herpes virus group by physicochemical and ultrastructural studies. Based on the data obtained, we had proposed the name herpesvirus aotus for this agent (3). Serological studies have clearly indicated that this herpesvirus is a new agent with no cross relationship to most of the well known members of this ubiquitous group. The presence of neutralizing antibodies from apparently normal owl monkeys (some with high titers 2 logs and over, Table II) strongly suggests that H. aotus is native to this species. Of 34 sera tested, 88% showed neutralizing titers.

Herpesvirus aotus has a limited cell host range. Another cell culture that was employed during the course of this study was goat synovial bursa cells. These cells are particularly susceptible to many herpes viruses

TABLE II. Neutralization Indices of Randomly Selected Owl Monkey Sera/H. aotus.[a]

Sera	Animal no.	NI	Sera	Animal no.	NI
2326	588-68[b]	1.0	5034	66-69	2.5
2659	628-69[c]	1.0	5035	69-69	1.5
2833	203-68[d]	0.0	5036	78-69	0.5
3073	63-69	1.0	5037	84-69	2.5
3974	64-69	3.0	4824	6-70	2.0
3075	67-69	0.5	4825	12-70	3.0
3078	68-69[d]	0.0	4826	14-70	1.0
3077	70-69	3.0	4827	97-70	1.0
3845	486-69	2.5	4828	98-70	2.0
3945	539-69	0.5	4829	99-70	2.0
4025	109-69	1.0	4830	100-70	1.5
5033	65-69	0.5	6662	268-69	0.5
6667	696-70	1.0	6657	25-70	0.5
6659	638-70	1.5	6656	637-70	1.0
6664	597-69	0.0	6660	24-70	0.5
6658	23-70	1.0	6666	396-69	1.0
6670	689-70	0.0	6669	625-70	1.0

[a] Serum dilution 1:5 tested in OMK cells.
[b] Animal from which H. aotus was isolated.
[c] Animal from which another virus (not herpes) was isolated.
[d] Animal from which another herpes virus was isolated.

TABLE III. Herpesvirus aotus Cytopathic Effect Compared with Other Herpes in Goat Synovial Bursa and Capsule.[a]

Virus	Cell Culture		
	SB	SBC	Inclusion
Herpesvirus simplex	3.5[b]	4.0	+
T	5.0	4.5	+
suis	5.5	4.5	+
B	4.5	5.5	+
SRNIA	6.5	7.5	+
Herpesvirus equine	2.5	3.5	+
canis	4.5	5.5	+
FRT	0	0	—
OMKI 372	0	0	—
Herpes aotus	0	0	—

[a] SB = synovial bursa; SBC = synovial bursa capsule; + = inclusion present; — = inclusion not present; SRNIA = sand rat nuclear inclusion agent; FRT = feline rhinotracheitis; OMKI 372 = owl monkey kidney isolate 372.

[b] Virus titer/ml.

as shown in Table III.

The presence of a latent agent like H. aotus in OMK ought to be considered when employing these cell cultures for experimental work, particularly when primary cell cultures are utilized without any observation period. The presence of these agents is likely to interfere with experimental data and may cause a severe setback if employed for the preparation of stock viruses.

These difficulties can be avoided by holding OMK primary cell cultures for at least 90 days before use in experiments. This long period of 90 days is suggested as we have recovered viral agents beyond 70 days (unpublished data). Fortunately these cell cultures endure long-term cultivation with changes of media as indicated by the pH. The media employed in our laboratory, Eagle's minimum essential medium with 10% fetal calf serum, has been particularly good for OMK cultures and we have been able to develop a cell line OMK 210-68, now in its 85th transfer.

Herpesvirus aotus has not been pathogenic to rabbits and goats and its infectivity spectrum is not fully known. Employment of owl monkeys and their cell cultures ought to be done with caution, since it is known that Herpesvirus B, a latent virus of rhesus monkeys, is highly infectious to man. Furthermore, another latent virus (H. saimiri) from the squirrel monkey has been shown to be oncogenic to other monkeys and rabbits (2, 15).

It is difficult to speculate how H. aotus is native to this species. How is the virus transmitted? Is there a vector and what other species of animals may carry this virus or antibodies against it, awaits further study.

Summary. An uninoculated batch of owl monkey kidney cell cultures yielded a viral agent 23 days after the cell culture was initiated. This agent possessed the physical, chemical, cytopathic, histological, and ultrastructural properties of a herpesvirus. Random testing of owl monkey sera showed the presence of high titered neutralizing antibodies against this new agent. Herpesvirus simplex, Herpesvirus T., Herpesvirus B, Herpesvirus suis, Herpesvirus saimiri, infectious bovine rhinotraecheitis, and Herpesvirus saguinus, OMKI 372, and OMKI 68-69 antisera failed to neutralize the infectivity of this new agent. In cell cultures the virus grew best in cells of owl monkey origin. It also grew (poorly) in cebus monkey kidney cell cultures, some batches of squirrel monkey kidney cells, in Vero, BSC-1, human embryonic lung, whole human embryo and human embryo skin and muscle cells. It failed to form plaques under an agar overlay and does not form pocks on chorioallantoic membranes of embryonated eggs. Based on these findings, the name Herpesvirus aotus has been suggested.

Owl monkey kidney cell cultures and owl monkey sera, when used for any work with other members of the herpesvirus group, ought to be employed with caution. The case reported here represents the first herpesvirus isolated from an owl monkey. Even though the pathogenic spectrum of this virus has not been established, being a herpesvirus, it should be regarded as potentially dangerous. Investigators who use owl monkeys should be aware of this new herpesvirus.

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