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Replication of Parainfluenza Type 3 Virus in Alveolar Macrophages: Evidence of In Vivo Infection and of In Vitro Temperature Sensitivity in Virus Maturation

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Approximately 2% of cultured alveolar macrophages (AM), originally lavaged from the lungs of parainfluenza type 3 virus (PI-3V)-infected calves, were observed to contain viral antigen (by fluorescent antibody method) or viral nucleocapsids (by electron microscopy). Plaque assays, however, indicated that virus titers were generally low when cultures were incubated at 37°C for 10 days. AM, obtained from "in vivo infected" and "noninfected" calves, were found to be equally susceptible to further in vitro PI-3V infection when cultures were incubated at 37°C. AM that were obtained from the lungs of normal calves, cultured at 37°C, and inoculated with PI-3V were observed to produce relatively high virus titers when the incubation temperature was shifted down to 32°C. Results from hemagglutinin assays showed that considerable amounts of hemagglutinin were detected when AM cultures were incubated at 32°C, but only limited amounts were detected at 37°C. Results from electron microscopic examinations at both temperatures substantiated the results of plaque and hemagglutinin assays. The PI-3V, isolated from AM cultures incubated at 32°C, grew well in Madin-Darby bovine kidney cells at 32°C, but little virus was produced at 37°C. In contrast, parent PI-3V grew equally well at both temperatures. The results are discussed in terms of host susceptibility, temperature-sensitivity and virus maturation, and surface viral antigens and persistent viral infection.

It is generally considered that alveolar macrophages (AM) constitute the primary phagocytic system against infectious microorganisms in the lung and play a major role in the host defense mechanisms against respiratory diseases. Although the defensive role of AM in bacterial infections has been well established (3, 4), the role of AM in viral infections is less well defined. Most studies have indicated that macrophages do not destroy viruses as they do certain bacteria, but the constant association of viruses with macrophages suggests that these cells are important in some manner in the pathogenesis of viral disease (5, 17).

In a previous study, we reported the viral pathogenesis of parainfluenza type 3 virus (PI-3V) infection in the bovine respiratory tract (20), but there was no evidence that AM could support PI-3V replication in vivo. Little is known about the effect of PI-3V upon AM; in particular, there are no ultrastructural data that concern the interaction between PI-3V and AM. Furthermore, little has been done to elucidate the function of AM after PI-3V infection with respect to their role as host cells for virus replication or in viral pathogenesis, in particular, viral persistence.

Macrophages, including AM, have been considered to be a factor in the development of persistent viral infections (9), but the mechanism(s) of their involvement is generally speculative. Another factor, which is often implicated in persistent viral infection, is the production of temperature-sensitive (ts) mutants, although the precise mechanisms responsible for the selection of ts virus during the evolution of persistent infection remain hypothetical (13).

The purpose of the present study was to explore at the ultrastructural level the cellular response of AM to PI-3V infection and to compare the hemagglutinin (HA) activity and virus yield of infected AM, which had been incubated at two different temperatures for 10 days. Specifically, we have examined AM cultures (i) from lungs of PI-3V-infected calves, (ii) from lungs of control calves, (iii) from (i) and (ii) that were inoculated with PI-3V in vitro, and (iv) that were PI-3V-infected in vitro and incubated at 37 and 32°C.

MATERIALS AND METHODS

Virus. A bovine strain of PI-3V described previously (19) was used. The inocula had infectivity titers from 3.7×10^7 to 2.3×10^9 plaque-forming units (PFU)

per ml. All virus inocula prepared from permissive cell cultures will be referred to as PI-3V hereinafter. Virus preparations obtained from infected AM will be referred to as PI-3V-AM.

Experimental calves. The colostrum-free calves of both sexes (2 to 18 days old at the beginning of the experiment) were obtained from the Research Station, Ontario Agriculture College, Elora, Ontario. The calves were cared for during the entire experiment as described previously (20).

Aerosolization of calves with PI-3V. The method of aerosolization of animals was described previously (20).

Experimental design. In the present study, AM were obtained from 12 colostrum-free calves, which were divided into four groups. Each group consisted of three calves: two infected and one control. Calves were sacrificed at 5, 7, 12, and 18 days postexposure, respectively. From each of the calves, half of the lungs, which include apical, cardiac, and diaphragmatic lobes, were removed aseptically for the procurement of the AM. Cells obtained from lung washing were used either directly for morphological studies or for AM culture and further in vitro inoculation of PI-3V.

AM culture. The procedures for collection and cultivation of AM, originally described by Myrvik et al. (10) for rabbits, were slightly modified for the bovine species. After the lungs had been washed thoroughly with sterile physiological saline and transected through the major bronchi, they were suspended by attaching hemostats to the wall of the bronchi, leaving the lumens open. The washing solution, which consisted of Hanks balanced salt solution, penicillin G potassium (500 IU/ml), and streptomycin sulphate (200 μg/ml), was introduced gradually via the bronchus. The lungs were gently massaged. The washings were collected by suction in a filtering flask (1,000 ml). The filling and washing of lungs were repeated several times, and the cells were sedimented by centrifugation at 800 $\times$ g for 30 min at 4°C. The pellets were suspended and pooled in growth medium that consisted of Eagle minimum essential medium in Hanks balanced salt solution supplemented with 20% fetal calf serum and double-strength antibiotics, as described for the washing solution. The fetal calf serum was heat inactivated (56°C, 30 min) and screened for the presence of anti-PI-3V antibody. Fetal calf serum that showed hemagglutinin (HA)-inhibition titers of 1:32 or higher was not used. The suspended cells were distributed proportionally into Leighton tubes, plastic flasks (Falcon, 250 ml), or Blake bottles and incubated at 37°C for 1 h. The majority of the AM adhered to the surface of the tubes or bottles within 1 h. The medium containing cell debris and mucous substances was discarded. The cultures were washed once with washing solution and once with growth medium, and the medium was then replaced with fresh growth medium. The cultures were incubated at the desired temperature.

Plaque assay. The plaque assay was carried out either in bovine embryonic kidney cells or in Madin-Darby bovine kidney (MDBK) cells purchased from Grand Island Biological Co., Grand Island, N.Y. The cultures were grown in 60-mm Falcon plastic tissue culture dishes in a 5% CO_2 incubator at 37°C. When monolayer cell sheets developed, each culture dish was inoculated with 0.5 ml of virus dilution (two dishes per dilution). After 1 h of adsorption at 37°C in a 5% CO_2 incubator, the cultures were overlaid with 0.5 ml of 0.8% warm Noble agar in Eagle minimal essential medium. The plates were further incubated under the same condition for 3 days, and plaques were counted after staining with carbol fuchsin.

Determination of HA activity. A microtest for HA activity was performed in plastic plates. Serial twofold dilutions of the material (0.05 ml in phosphate-buffered saline [PBS]) were set up; 0.05 ml of a 0.5% suspension of bovine erythrocytes in PBS was then added to each dilution. Readings were taken after 4 h at 37°C when sedimentation was apparent. The last well exhibiting a bottom pattern of clear partial agglutination was considered to contain 1 HA unit.

Light microscopy (LM) and fluorescent microscopy. AM were fixed in situ on cover slips with Bouin solution or 1% glutaraldehyde at appropriate intervals after infection and were stained with hematoxylin and eosin. The direct method of fluorescent microscopy was used as described previously (19). Briefly, the infected and control cultures were fixed at appropriate intervals in cold acetone, dried in air, and treated with fluorescent isothiocyanate-conjugated antibody (goat generated, Colorado Serum Lab., Denver, Colo.). The cells were then washed three times with PBS, mounted in buffered glycerine, and examined with a Zeiss fluorescent microscope.

Transmission electron microscopy (EM). The preparation of thin sections has been previously described (19), except for the following modification. After low-speed centrifugation, the pelleted macrophages were gently resuspended in 1.0 ml of PBS. About 0.25 ml of suspended cells was distributed into each bottleneck polyethylene capsule and centrifuged for 10 min at 800 $\times$ g. After the removal of the supernatant fluid, the resulting pellets were fixed in situ with 2.5% buffered glutaraldehyde for 30 min, and the fixative was removed and replaced by sucrose washing buffer.

Scanning electron microscopy (SEM). AM were fixed in situ on glass cover slips with glutaraldehyde, which was added to the medium in the Leighton tubes to a final concentration of 1.0%, and the macrophages were fixed for 1 h at room temperature. In all subsequent steps, care was taken to keep the surface of the cover slip under fluid to prevent cell distortion. The medium containing glutaraldehyde was replaced with 1.0% (glutaraldehyde phosphate buffer, pH 7.3), and the macrophages were held at 4°C for 1 to 7 days. Fixed monolayers were gradually dehydrated in serial 15-min changes of 30, 50, 70, 95, and 100% ethyl alcohol, followed by two 15-min treatments with amyl acetate. The amyl acetate was replaced by liquid CO_2 in a Polaron critical point drying apparatus. The CO_2 was brought to the critical point, and the gaseous CO_2 was released. The specimens were then coated with a layer of gold or gold and palladium in an evaporator with a rotary coater arrangement and were observed with an SEM at 25-kV accelerating voltage.

RESULTS

PI-3V infection in AM in vivo. A previous report (20) concerning PI-3V infection in the bovine respiratory tract has raised the important

question of whether AM support PI-3V replication in vivo. To answer this question, AM obtained from PI-3V-infected calves were studied as follows.

(i) Assay of viral antigen by microscopy. AM harvested from PI-3V-infected calves 5 to 18 days postinfection were cultured in vitro for 10 days. At various intervals, cultures were removed and examined by LM, fluorescence microscopy, and EM.

By LM, irregularly shaped, eosinophilic inclusion bodies could be seen in the cytoplasm of a few AM or of small multinucleated cells (Fig. 1A). Intranuclear inclusions were occasionally visible (Fig. 1A). The majority of AM, however, appeared normal with prominent ruffles and pseudopods (Fig. 1B). Under SEM, the surface structures, characteristic of macrophage morphology, could be vividly seen as ruffles, microvilli, and slender filopodia (Fig. 2A). About 5% of the AM population showed various changes in surface structures when AM cultures were examined by SEM. These changes included the lack of slender filopodia and the partial or complete disappearance of ruffles (Fig. 2B).

Further evidence that AM were infected by PI-3V in vivo was provided in the present study. This was demonstrated by the presence of viral antigen, revealed by using the fluorescent antibody method (Fig. 2C), and by the presence of viral nucleocapsids (VN) in the cytoplasm of AM, indicated by thin section EM (Fig. 3).

The percentage of AM that contained viral antigen was slightly higher than the percentage of AM that showed VN (Table 1). This was probably an indication that only limited areas were examined by EM. In calves that were infected with PI-3V and sacrificed between 5 to 7 days postexposure, 2.7 to 3.5% of AM appeared to be infected by PI-3V when AM were cultured for 3 to 10 days in vitro. By 18 days postexposure in vivo, the percentage of AM containing viral antigen dropped to 0.5 to 1.3%, and VN were no longer detectable by EM. The percentage of infected AM did not increase when the cultures were maintained in vitro for the next 7 days (10 days from the beginning of cultures) at 37°C.

By fluorescent microscopy, the introcytoplasmic inclusions were seen as being very small granules (Fig. 2C). Some of the inclusion bodies had a somewhat diffuse outline. In general, the coalesced inclusion bodies observed in the PI-

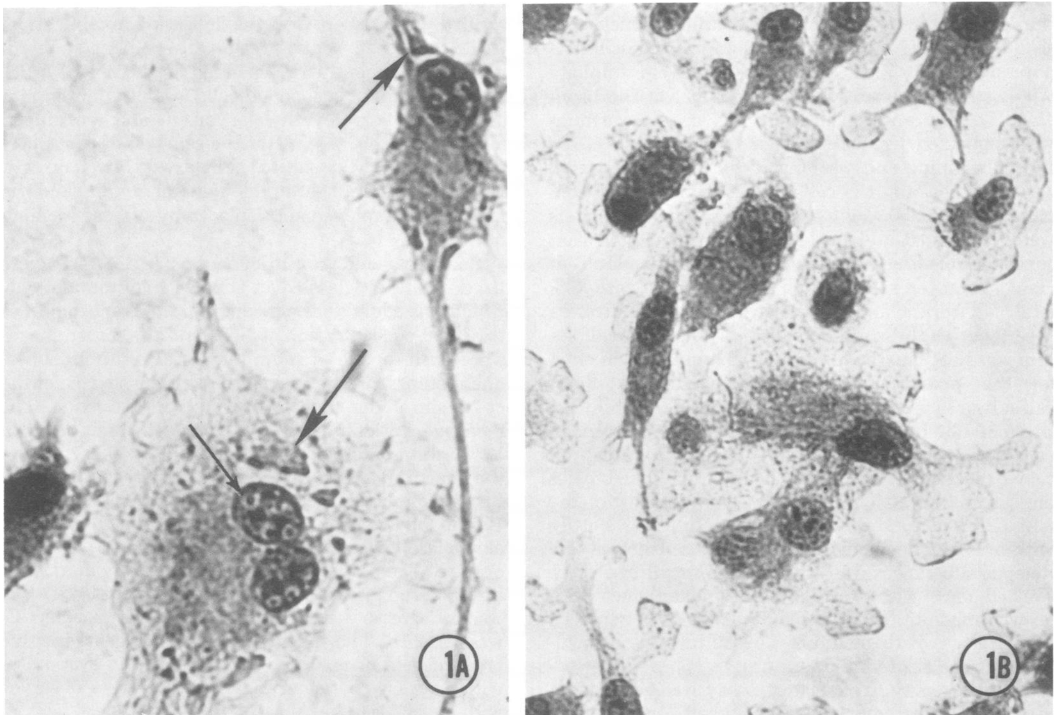

FIG. 1. *(A) LM of AM from the lung of a PI-3V-infected calf (5 days postinfection) cultured for 3 days in vitro. Note the intracytoplasmic (large arrows) and intranuclear (small arrows) inclusions in the mononuclear and binucleated cells. ×1,940. (B) LM of AM from the lung of a PI-3V-infected calf (7 days postinoculation) cultured for 3 days in vitro. Note the prominent ruffles and pseudopods. ×1,340.*

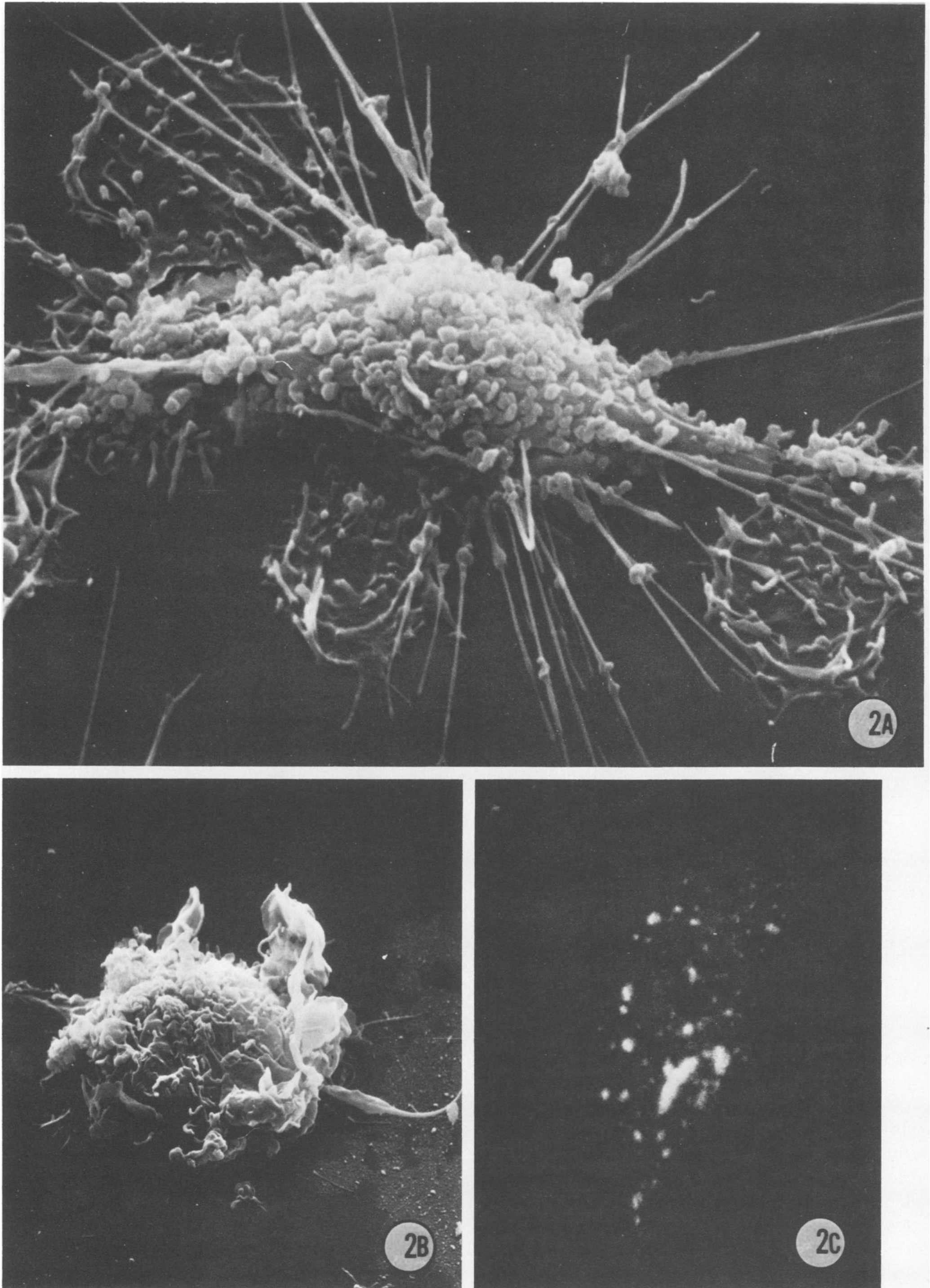

FIG. 2. *(A) SEM of a normal AM cultured as described in the legend to Fig. 1A. Note the prominent surface structures, ruffles, microvilli, and slender filopodia characteristic of macrophage morphology. ×7,800. (B) SEM of an AM cultured as described in the legend to Fig. 1A. Note the total lack of slender filopodia and the partial disappearance of ruffles. ×4,000. (C) Fluorescent micrograph of AM cultured as described in the legend to Fig. 1A. Intracytoplasmic viral antigen was detected as small granules. ×2,500.*

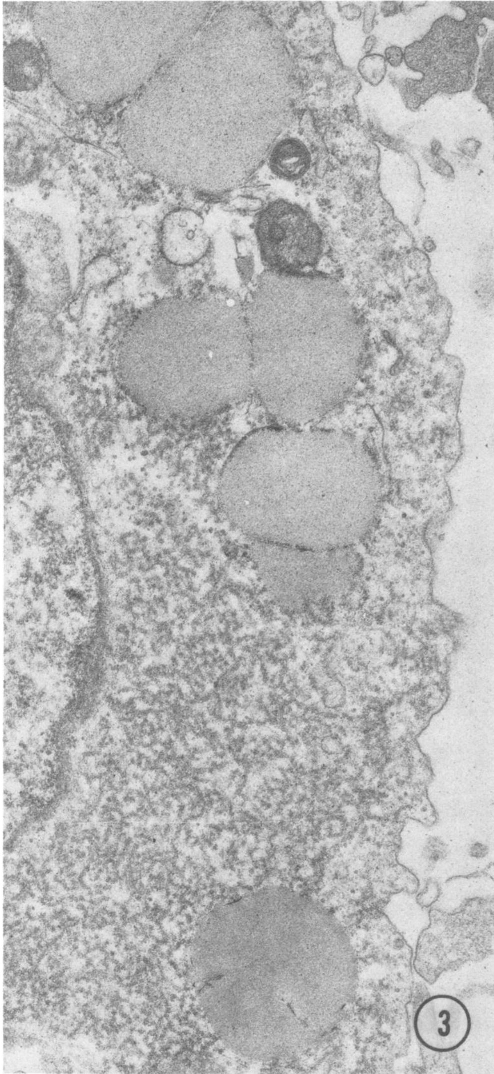

FIG. 3. *EM of portions of AM showing extensive accumulations of VN in the cytoplasm near the cell surface but the absence of virus buddings at the cell surface. ×18,900.*

3V-infected embryonic kidney cells (19) were rarely noted in the infected AM, even when the incubation time was extended to 10 days.

At the ultrastructural level, the most prominent change observed was the accumulation of VN in the cytoplasm of infected AM (Fig. 3). The viral nucleocapsids consisted of random masses of intertwined tubules, which in some cases were difficult to distinguish clearly, since they manifested a fuzzy appearance. Virus budding processes or isolated virus particles were not identified in those AM that contained viral nucleocapsids when they were further cultured in vitro for 3 to 10 days at 37°C.

(ii) Plaque assay. To determine whether infectious virus was synthesized in each of the AM cultures, cells at various intervals were separated from culture fluids by centrifugation at 800 × *g* for 20 min. The cell pellets were suspended in 3.0 ml of PBS. The culture fluids and cell suspensions were used for subsequent plaque assay on MDBK cells. Immediately before plaque assay, the cell suspension was sonically treated for 30 s in an ice bath. After low-speed clarification, the supernatant fluids obtained from cell suspensions were referred to as "cell-associated material." Except in two instances (A2, 3 days in culture, and D3, 6 days in culture), no infectious virus could be recovered from the culture fluids, although low virus titers were generally detectable in the cell-associated material (Table 1).

PI-3V infection in AM in vitro at 37°C. AM obtained from PI-3V-infected and normal control calves were cultured for 1 to 2 days and further inoculated with PI-3V. At intervals after seeding, cultures were removed for microscopic examinations, plaque titrations, and HA assays. The experiments were designed to determine whether there was a difference in susceptibility to PI-3V between AM from infected and noninfected calves.

(i) Assay of viral antigen by microscopy. There was no marked difference in susceptibility to in vitro inoculation of PI-3V when AM from infected and noninfected calves were compared (Table 2). The durations of 5, 12, and 18 days were chosen because at day 5 postexposure extensive lung lesions were found (20), and at days 12 and 18 early recovery stages occurred in which lung macrophage function might be active in combating viral infection.

When the percentages of multinucleated giant cells between in vitro infected groups (B2, B3, E2, and E3 of Table 2) and in vivo infected groups (A2, A3, D2, and D3 of Table 1) were compared, a substantial increase in that of the former groups was evident. These giant cells contained more nuclei and often had intranuclear inclusions (Fig. 4).

The percentage of infected AM determined by the fluorescent antibody method was higher than that determined by LM and EM. The percentage of infected AM gradually declined as the duration of in vivo infection increased, but no marked difference was noted between the infected and noninfected ones in each set of cultures (Table 2).

Ultrastructurally, the presence of VN in the cytoplasm of AM was used as a criterion, as

TABLE 1. *Evidence of PI-3V infection in AM in vivo*

AM culture	In vivo infection (days)	In vitro culture at 37°C (days)	Giant cell with intracytoplasmic inclusion (mean %)	Cell positive with viral antigen (FA)[a] (mean %)	Cell positive with VN (EM) (mean %)	Virus titer	
						Cell-associated material (0.1 PFU/ml)	Culture fluid (0.1 PFU/ml)
A1[b]	5	1	ND[c]	ND	ND	2.3×10^2	0.0×10^1
A2	5	3	1.5	2.7	1.5	3.5×10^2	2.0×10^1
A3	5	6	2.0	3.5	1.5	2.8×10^2	0.0×10^1
A4	5	10	2.2	3.0	ND	4.0×10^1	0.0×10^1
D1	7	1	ND	ND	ND	3.0×10^2	0.0×10^1
D2	7	3	1.2	2.8	1.0	1.6×10^2	0.0×10^1
D3	7	6	2.0	3.0	1.5	6.0×10^1	1.3×10^1
D4	7	10	1.8	3.5	ND	ND	ND
G1	18	1	ND	ND	ND	0.0×10^1	0.0×10^1
G2	18	3	0.5	1.0	0.0	0.0×10^1	0.0×10^1
G3	18	6	1.3	1.1	ND	ND	ND
G4	18	10	0.5	0.5	ND	ND	ND

[a] FA, Fluorescent antibody method.
[b] Calves A, D, and G were infected with PI-3V and sacrificed at 5, 7, and 18 days postexposure, respectively.
[c] ND, Not determined.

TABLE 2. *Incidence of PI-3V infection in AM cultures at 37°C*

AM culture	In vivo infection (days)	In vitro infection (days)	Giant cell with inclusion bodies (mean %)	Cell positive with viral antigen (FA)[a] (mean %)	Cell positive with VN (EM) (mean %)
B2[b]	5	2	6.8	12.5	4.8
B3	5	5	9.2	21.7	7.3
C2	0	2	5.3	10.1	5.1
C3	0	5	13.6	7.4	6.8
E2	12	2	6.3	9.0	3.8
E3	12	5	9.5	12.8	ND[c]
F2	0	2	5.6	10.3	5.2
F3	0	5	7.0	10.6	ND
H2	18	2	3.3	6.5	ND
H3	18	5	4.0	5.8	3.5
J2	0	2	3.9	8.6	ND
J3	0	5	4.5	8.8	2.6

[a] FA, Fluorescent antibody method.
[b] Calves B, E, and H were infected with PI-3V and sacrificed at 5, 12, and 18 days postexposure, respectively. Calves C, F, and J were sacrificed at the same time as calves B, E, and H, respectively, for control purposes.
[c] ND, Not determined.

described above, for positive PI-3V infection. Two features, which were not observed in the previous in vivo groups, were noted. First, the VN were often seen partially (Fig. 5) or completely enclosed by unit membranes. The enclosed VN appeared to be altered, and electron-dense material was often seen intermingled with them. Second, free VN were occasionally seen in the extracellular areas near the cell cytoplasm. Virus budding processes were generally absent.

(ii) Plaque assay. Samples for AM cultures infected in vitro with PI-3V at days 2 and 5 postexposure (Table 2) were examined by plaque assay for the presence of infectious virus. The results substantiated microscopic observations that (a) AM obtained from "in vivo infected" and "noninfected" groups were equally susceptible to in vitro PI-3V infection, and (b) low virus titers were generally noted in cell-associated samples, and little or no virus was detected in culture fluids.

(iii) HA assay. Weak HA activity was detected in the cell-associated material, and extremely low HA activity was measured in the culture fluids (Fig. 9B).

Cultivation of AM at 32°C. In an attempt to maintain our AM cultures longer, we found that temperature and pH were critically important. These, of course, are not unique requirements for AM. Between 31 and 33°C and with a slightly acidic pH, AM cultures could be maintained for much longer periods. At 37°C AM spread well on the surface with prominent ruffles and pseudopods, whereas at 32°C AM showed less cytoplasmic processes, but they still adhered well to the surface.

Isolation of infectious virus from AM cultures—temperature shifting experiments. AM obtained from colostrum-free calves were initially cultured at 37°C. On day 2 of culture, cells were inoculated with PI-3V at an input multiplicity of 1 PFU/ml. Half of the cultures were shifted and incubated at 32°C, while the other half were retained at 37°C. At intervals, culture fluids and cells were harvested separately and treated further as described above. These materials were assayed for infectivity and HA activity. In addition, at days 3, 6, and 10,

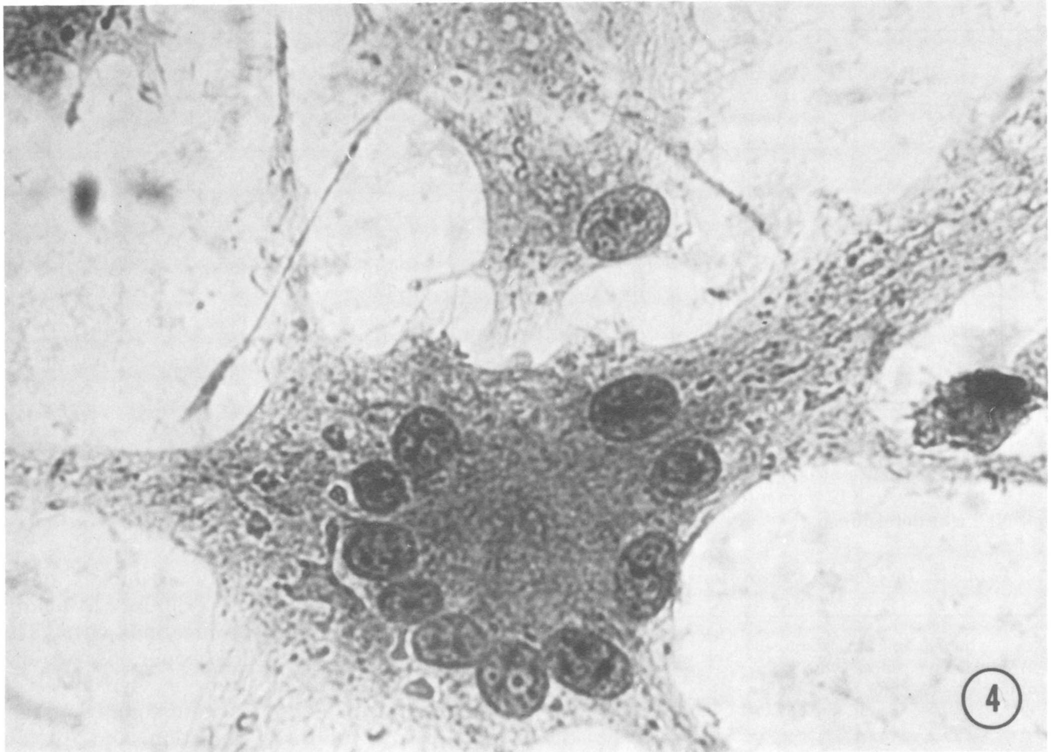

FIG. 4. *LM of AM lavaged from the lung of a PI-3V-infected calf (12 days postinfection). Cultured in vitro for 1 day and further inoculated with PI-3V (5 days postinoculation). Note the multinucleated giant cell formation with intracytoplasmic and intranuclear inclusions. ×1,940.*

after shifting the temperature, infected AM were harvested and processed for EM examinations.

(i) Assay of virus infectivity by EM. In addition to the presence of VN in the cytoplasm of AM (Fig. 6), as described above, an unusual virus budding process was observed in the infected AM when cultures were incubated at 32°C. The virus budding was expressed by (a) the protrusion of cytoplasmic membranes, (b) the presence of VN immediately beneath the protruded membrane, and (c) the accumulation of internal electron-dense material near the nucleocapsids; there was little or no external coating material either at the protruding membrane or along the cell surface (Fig. 7A and B).

The external coating material (site of HA and neuraminidase), however, could be observed rarely in the intracellular vacuoles in which virus particles matured through the cytoplasmic membrane and remained cell associated. These observations thus substantiate the results of HA assays, which are described below.

(ii) Plaque assay. Relatively high virus titers could be detected in the culture fluids and cell-associated materials when AM cultures were incubated at 32°C (Fig. 8A). The virus titers reached a plateau about 3 days postinfection. The cell-associated material showed virus titers about 1 log higher than that of the culture fluids at days 3 and 6 postexposure. By day 10 postexposure, the virus infectivity of cell-associated material was retained, while the virus titer of the culture fluids declined considerably. In infected AM incubated at 37°C, low virus titers were obtained from all cell-associated material, and little virus or no plaque was detected in the culture fluids (Fig. 8B).

(iii) HA assay. When AM cultures were incubated at 32°C, a considerable amount of HA activity was detected in the cell-associated material as well as in the culture fluids (Fig. 9A); whereas at 37°C, only a limited amount of HA was detected in the cell-associated material and there was almost no HA activity in the culture fluids (Fig. 9B).

Growth of PI-3V-AM and PI-3V in MDBK cells at 32 and 37°C. The PI-3V virus isolated from AM cultures incubated at 32°C was designated as PI-3V-AM. To examine the growth of PI-3V-AM, MDBK cells were infected with an input multiplicity of 1 PFU/ml. After 1 h of adsorption at 32°C, monolayers were washed

and incubated separately at 32 and 37°C. At intervals, culture fluids were removed from the monolayers and assayed for infectious virus by the plaque method. Concurrently, MDBK cells were infected with PI-3V at the same input multiplicity for comparative purposes. PI-3V-AM grew well at 32°C, but little virus was produced at 37°C (Fig. 10A). In contrast, PI-3V grew equally well at 32 and 37°C (Fig. 10B).

DISCUSSION

The evidence that AM are indeed infected by PI-3V in vivo is provided in the present study. This conclusion was based on microscopic examinations and plaque assays of AM obtained from PI-3V-infected calves between 5 and 18 days after infection and cultivation in vitro for 1 to 10 days. Infection was demonstrated in these in vivo, PI-3V-infected AM by (i) the formation of multinucleated giant cells with intracytoplasmic inclusions, (ii) the presence of viral antigen, indicated by use of the fluorescent antibody method, and (iii) the presence of VN in the cytoplasm, indicated by the use of transmission EM.

Although SEM showed that about 5% of the AM population had various surface modifications, there was no evidence that they were directly related to PI-3V infection.

Although infected AM showed relatively large amounts of VN (an indication of virus replication), it was extremely difficult to identify virus budding processes or isolated, extracellular virus particles when AM cultures were incubated at 37°C. These observations were further supported by the plaque assay data, which verify that, from the cell-associated material and two of the culture fluids, low levels of virus were recovered. These findings (both microscopic and plaque assays) may explain why the percentage of infected AM did not increase significantly when the cultures were maintained in vitro for the next 7 days at 37°C.

Seemingly, at 37°C, the mode of PI-3V replication in AM differs from that previously observed in bovine embryonic cell cultures (19). PI-3V replication in our AM cultures at 37°C

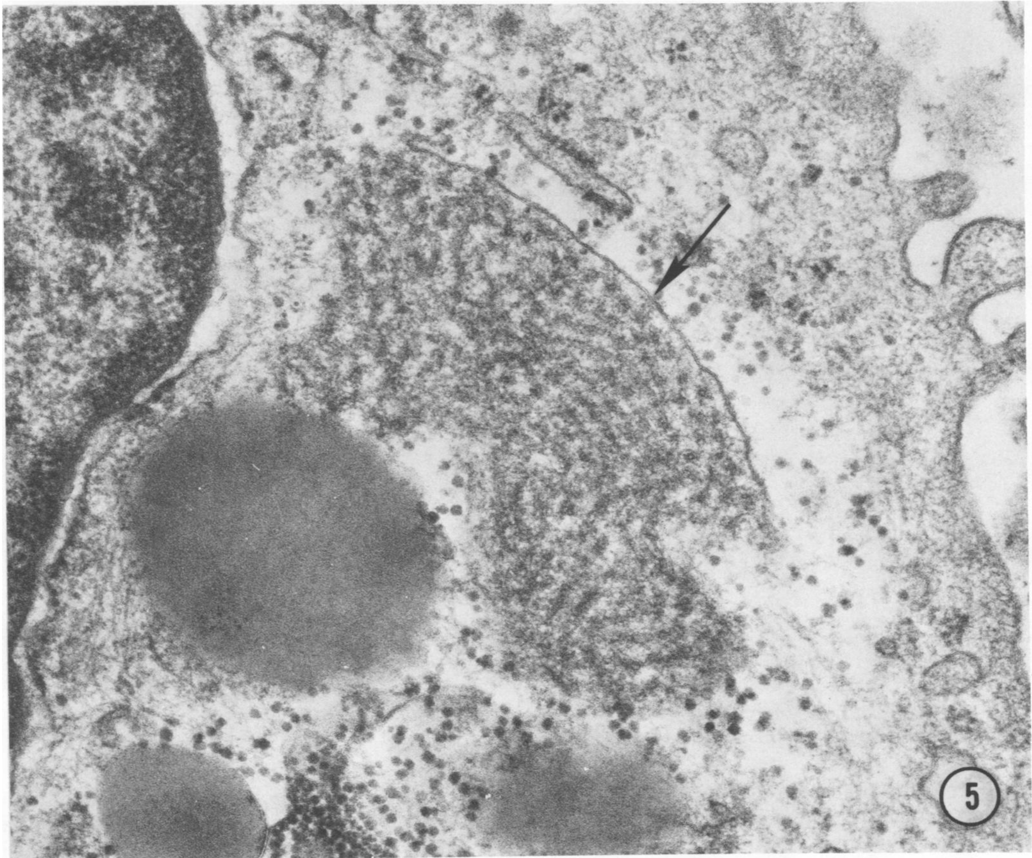

FIG. 5. *Portion of a PI-3V-infected AM showing VN partially enclosed by a unit membrane (arrow). ×59,500.*

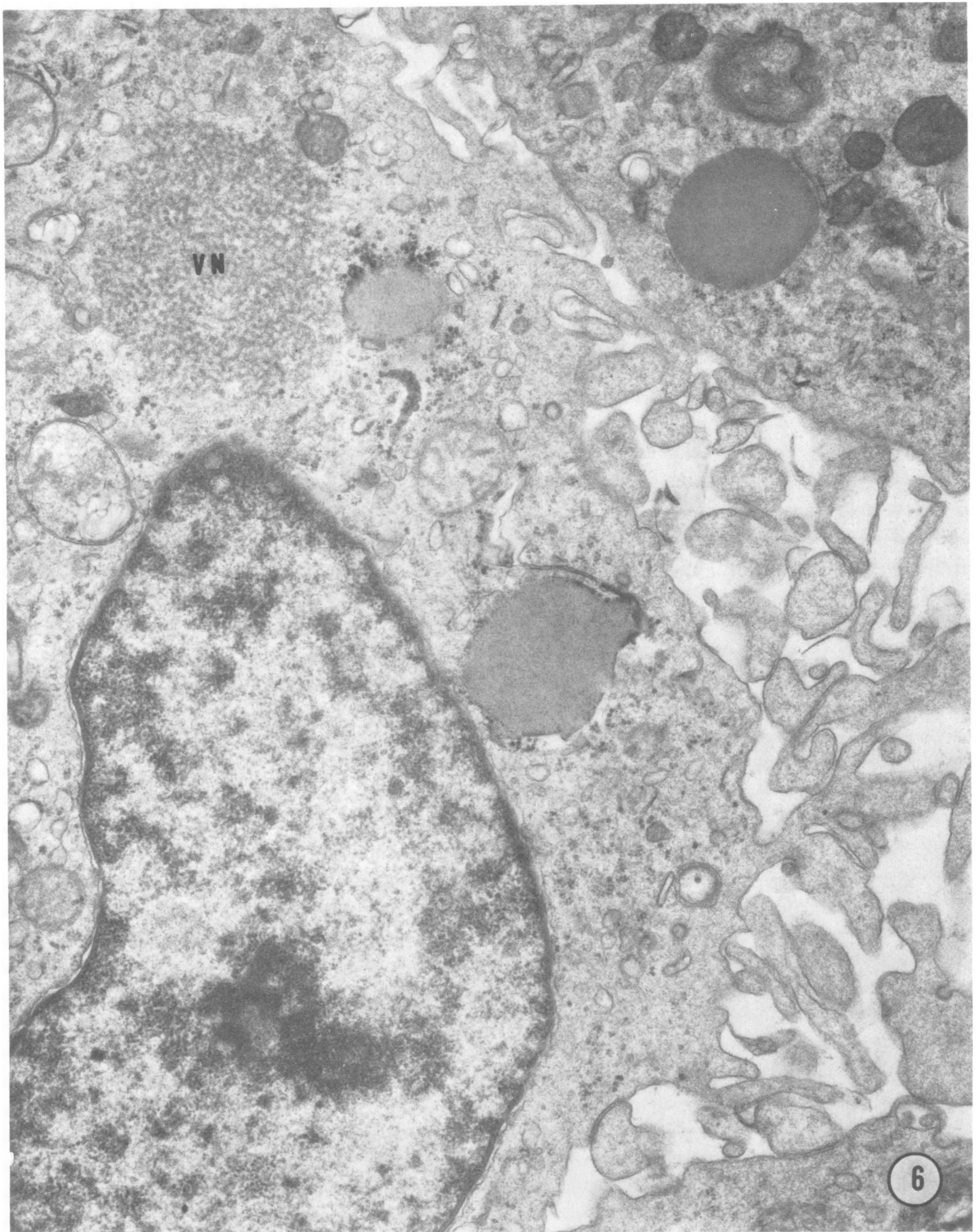

FIG. 6. *EM of an AM inoculated with PI-3V at 37°C, shifted to 32°C for incubation, and harvested 3 days later. Note the VN in the cytoplasm. ×21,800.*

appeared to be defective at the later stages of replication. This conclusion is based on observations that considerable amounts of VN were regularly found in the cytoplasm of AM and infectious virus particles were seldom demonstrable. This phenomenon is somewhat similar to latent subacute sclerosing panencephalitis viral infection in which VN were frequently

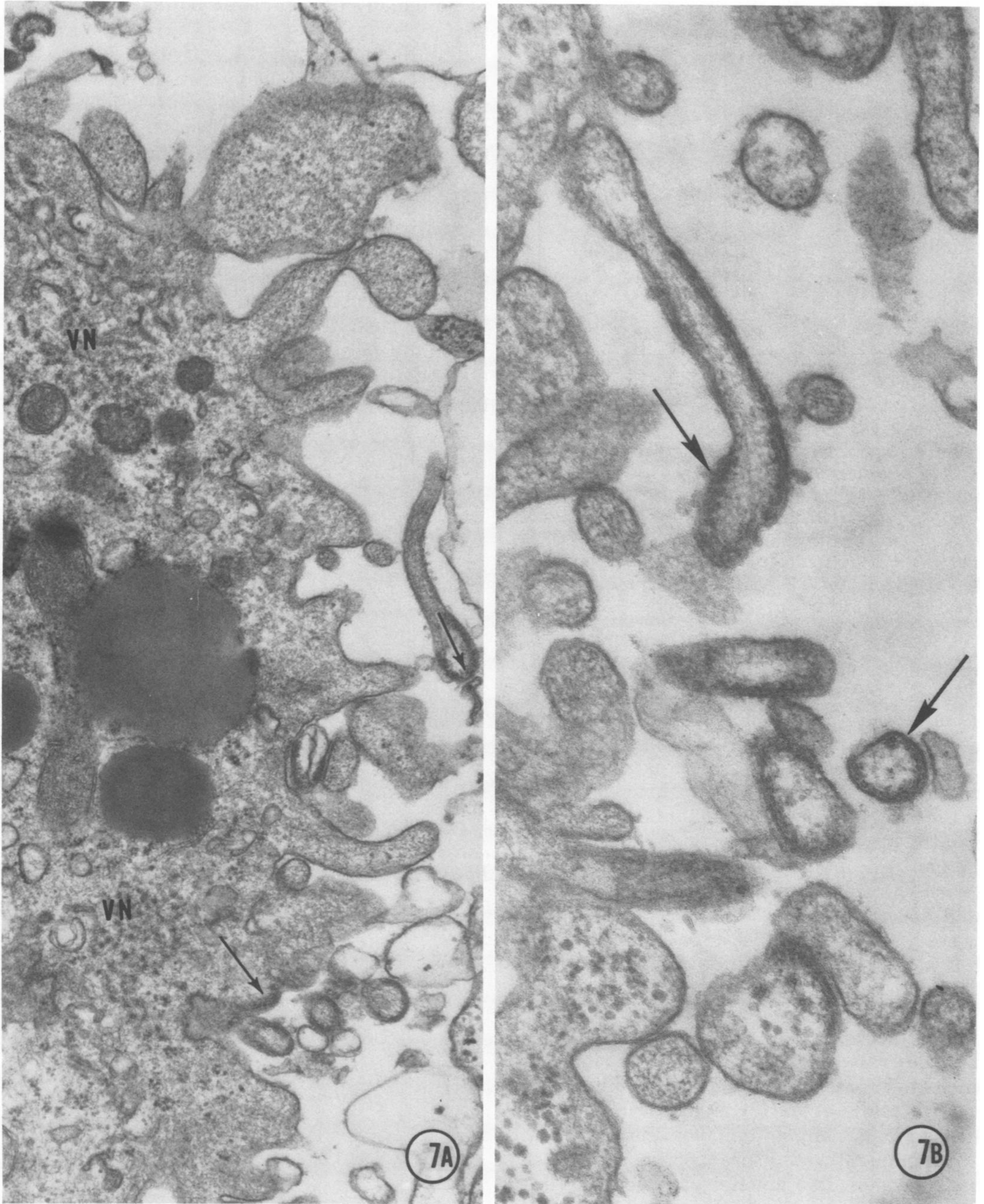

FIG. 7. *(A) EM of an AM inoculated with PI-3V at 37°C, shifted to 32°C for incubation, and harvested 6 days later. Note the scattered VN in the cytoplasm, the VN immediately beneath the protruded membrane (arrows), and the absence of external coating material. ×26,700. (B) EM of a portion of an AM inoculated with PI-3V at 37°C, shifted to 32°C for incubation, and harvested 6 days later. Note the virus buddings (arrows) expressed by the protrusion of cytoplasmic membranes, the VN immediately beneath the protruded membrane, the accumulation of internal electron-dense material surrounding the VN, and the lack of external coating material. ×65,100.*

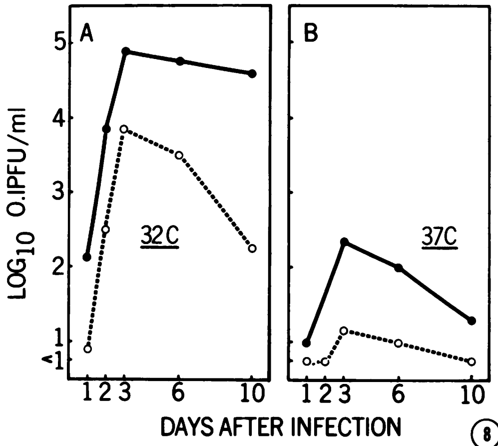

FIG. 8. *Growth of PI-3V in AM cultures at 32 and 37°C. The cultures were inoculated with PI-3V at an input multiplicity of infection of 0.1 PFU/ml. After 1 h of absorption at 37°C and addition of maintenance medium, cultures were incubated at 32 and 37°C. At intervals after infection, the cell-associated material* (●) *and culture fluids* (O) *were assayed for virus infectivity.*

found in abundance, but VN antigen is not assembled into its complete virions (2). Whether the failure of VN assembly is related to some substance in AM that directly interferes with VN assembly or whether there is a failure of production of some viral component necessary for the assembly of VN could not be determined from the present study.

When AM obtained from PI-3V-infected and normal control calves were cultured and inoculated with PI-3V in vitro, no marked difference in susceptibility to PI-3V between the two groups was observed. These findings indicate that AM obtained from in vivo infected calves at 5 to 18 days did not show increasing resistance to viral infection. Thus, these data contrast the marked resistance of macrophages from immune rabbits to vaccinia infection (1, 15). Similar studies with rabbit AM (18) and bovine AM (14) failed to detect any difference in susceptibility.

Our temperature shifting experiments showed that a considerable amount of infectious virus could be demonstrated and a greater amount of HA activity could be detected in both culture fluids and cell-associated material. These data seem to indicate that PI-3V-infected AM may contain a complete set of viral genome that are only expressed fully at lower temperatures. A similar phenomenon has been reported in certain carrier cell lines in which reduction of temperature caused an increase in virus yield, although, in contrast to our data, HA appeared to be equally detectable at both temperatures (7).

The fluctuation of HA activity observed in the present study is of interest in view of the suggestion that infected cells with less or insufficient viral antigen on the surface may escape the attention of immunological surveillance in vivo (8). Fluctuation of surface antigen has also been described in cultured cells from the brain of a patient with subacute sclerosing panencephalitis (11). Mims (9) pointed out that a sufficient density of surface antigen is required if the antibody is to eliminate an infection by reacting with the surface antigen(s) and lysing the cell. When the density of viral antigen is inadequate, antibody-mediated lysis may not take place and persistent infection may be initiated (9). Our

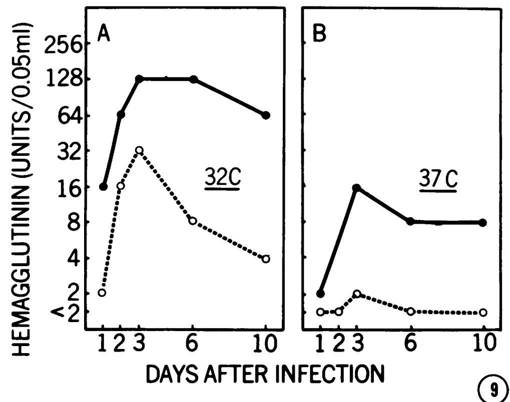

FIG. 9. *HA activity in cell-associated material* (●) *and culture fluids* (O) *of AM cultures infected with PI-3V and incubated at 32 and 37°C.*

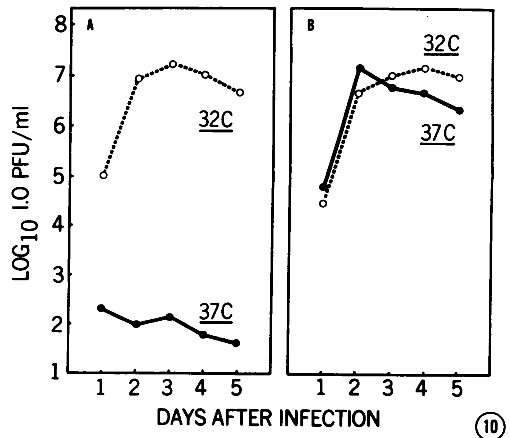

FIG. 10. *Growth curves of PI-3V AM (A) and PI-3V (B) in MDBK cells at 32 and 37°C. After 1 h of absorption at 32°C, monolayers were incubated separately at 32 and 37°C. At intervals, culture fluids were removed and assayed for virus infectivity.*

ultrastructural observations and HA assays favor this contention.

The mechanism(s) by which a persistent infection evolves in the host is generally unknown. The development of chronic infection may be due to either virus alteration, cellular selection, or both, and the mechanism of its induction very likely varies from one disease to another (9). One of the frequently considered factors in the evolution of persistent infection is virus infection of macrophages (9). For example, a wide variety of viruses, including adenoviruses, various herpesviruses, visna virus, measles virus, and certain reoviruses in mice infect macrophages (9); however, little information is available concerning the virus-macrophage interaction, especially at the ultrastructural level. The interaction between PI-3V and AM observed in the present study supports the view that cellular factors may play a role in the evolution of persistent viral infections.

Although there is growing evidence that ts mutants may be involved in the development of in vitro persistent infections of paramyxoviruses (6, 7, 12), the precise mechanism responsible for the selection of ts virus during the course of establishment of persistent infection remains unknown. It is, however, interesting to note that ts virus mutants have been isolated from a variety of carrier cultures of various host cell-virus systems (6, 12, 16).

An interesting finding in the present study is the emergence of a ts variant of PI-3V from infected AM. Temperature sensitivity in this report strictly refers to the fact that virus maturation is temperature dependent. Our data indicate that the lower temperature (32°C) at which the AM cultures were maintained favors the evolution of ts variant of PI-3V. Under this condition, PI-3V was not only capable of infecting AM, but also of producing virus particles without obvious destruction of the host cell. This nondestructive infection and the production of mainly cell-associated virus suggest the possibility that PI-3V in nature might produce covertly infected AM, and that if the condition is favorable (lower temperature), mature virus particles may be synthesized from the infected AM. It can be suggested further that this phenomenon may result in the persistence of residual virus in the respiratory tract.

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