Expression of Herpesvirus in Adherent Cells Derived From Bone Marrow of Latently Infected Guinea Pigs

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The role of bone marrow adherent cells in the latency of guinea pig herpes-like virus (GPHLV) was explored. Cultures of macrophage-enriched adherent cells derived from infected guinea pigs were examined for evidence of latent GPHLV infection. Expression of the virus was detected in these cultures 9 to 10 days after *in vitro* cultivation. Increasing virus infectivity titers as well as light and electron microscopic evidence of virion assembly in macrophages and fibroblasts were demonstrated. Infectious virus was detected in the bone marrow adherent cells that had attached for 30 or 120 minutes but only following reverse cocultivation. The data showed not only that the bone marrow adherent cells were susceptible to GPHLV *in vitro* but also that GPHLV was harbored by the macrophage-enriched bone marrow population *in vivo* in latently infected guinea pigs. (Am J Pathol 91:483–496, 1978)

A HERPES-LIKE VIRUS has been repeatedly isolated from tissues of latently infected guinea pigs without clinical disease. However, benign reactive hyperplasia of lymphoid tissues was observed following experimental infection.[1] Although infectious virus could not be demonstrated directly in various tissues taken from infected animals, the virus could be recovered following cultivation or cocultivation of the infected tissue cells.[2] The highest virus titers were usually obtained from organs of the lymphoreticular system.[3,4] These findings suggest that the cells carrying the latent viral genome are ubiquitous cells related to that system. Since the bone marrow is the source of many migrant cells in the lymphoreticular system, attempts were made to study the persistence of the guinea pig herpes-like virus (GPHLV) in cell populations of the bone marrow. More specifically, bone marrow macrophage-enriched populations, obtained as adherent cells, were maintained in culture for relatively long periods to allow the expression of the latent GPHLV. The results obtained, together with the growth characteristics and identification of guinea pig bone marrow adherent cells, are included in the present report.

Materials and Methods

Virus Strain

The LK40 strain of GPHLV was originally isolated from the spleen of a leukemic guinea pig[5] and was used throughout the present study.

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Accepted for publication February 6, 1978.

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0002-9440/78/0608-0483$01.00

Cell Culture for Virus Assay

Guinea pig embryo fibroblast (GPEF) monolayer cell cultures were used for virus assay. These cultures were prepared from 30- to 40-day-old fetuses of normal healthy Hartley guinea pigs and were grown in Eagle's basal medium with 10% fetal bovine serum as described previously.[6] For virus assay, the following procedures were used: a) The infectivity titers of virus in tissue culture suspensions were determined by inoculating serial tenfold dilutions into GPEF monolayer cultures. b) The infectivity titers of latent GPHLV in bone marrow cells, derived from infected guinea pigs, were determined by cocultivation of serial 10-fold dilutions of marrow cell suspensions with GPEF monolayer cultures. c) Detection of cell-free virus in marrow cell suspensions was determined by inoculation of marrow cell lysates which had been frozen and thawed three times into GPEF monolayer cultures. Virus infectivity titers were calculated according to the Reed and Muench method.

Animal Inoculation

Virus-free Hartley guinea pigs were inoculated intraperitoneally with 5×10^7 $TCID_{50}$ of GPHLV stock to establish infection. To insure that the animals were infected, blood samples from each animal were inoculated into GPEF monolayer for virus-induced CPE prior to use in the following experiments.

Bone Marrow Cell Cultures

Bone marrow flecks were taken from the long bones or the extremities of infected and noninfected guinea pigs. Marrow cells were disaggregated by forced pipetting and were suspended in medium RPMI 1640. Final cell counts were made from a 10% (v/v) stock marrow cell suspension according to the experimental design, as indicated in the text. Marrow cells were allowed to adhere to 22 × 11-mm glass coverslips or 60 × 15-mm plastic tissue culture dishes for various periods, washed with Hanks' balanced salt solution (HBSS), and grown in medium RPMI 1640 containing 20% fetal bovine serum.

Cell Characterization

Latex Particle Phagocytosis

Latex particles, 0.8 μ in diameter, were added to marrow cell suspensions or monolayer cultures. After 1 hour of incubation at 37 C, free latex was removed and the preparations were examined for detection of particle engulfment by macrophages under light and/or electron microscopy.

Nonspecific Esterase Reaction (NEE)

This cytochemical stain, as described by Yam et al[7] and modified by Kaplow, was used as an indicator of macrophages. Briefly, a mixture of α-naphthylacetate and fast blue BB salt was added to cells on coverslips previously fixed in buffered formalin acetone. In the presence of nonspecific esterase activity, the substrate was cleaved and the resulting free naphthyl radical was coupled with the fast blue BB salt to form a gray-black reaction product. This preparation was then counterstained with Giesma.

Light Microscopy

Marrow cells on coverslips were fixed in Zenker's acetic fluid and stained with hematoxylin and eosin or were fixed in methanol for Giemsa staining.

Table 1—Cultivation of Adherent Cells Derived From Bone Marrow of Guinea Pigs With or Without GPHLV Infection

Source of guinea pigs	No. of animals studied	Morphology of cultivated adherent cells		
		1-3 days	4-10 days	10-15 days
Noninfected	10	Mostly polygonal cells (promonocytes, monocytes, and macrophages)*	a) Stationary number of macrophages	Heterologous population of macrophages and spindle cells
			b) Increasing number of spindle cells (fibroblast-like)†	
GPHLV-infected	12	Same	Same	Macrophages and spindle cells showing CPE, inclusion bodies, and virions (light and electron microscopy)

* NEE (+), latex ingestion (+)
† NEE (−), latex ingestion (−)

Electron Microscopy

Marrow cells obtained from animals were allowed to grow in plastic tissue culture dishes in a CO_2 incubator for various periods or until virus-induced CPE appeared. Islets of cells were fixed *in situ* with 2% glutaraldehyde for 2 to 4 hours, postfixed with osmium tetroxide, embedded in Epon, and thin-sectioned for electron microscopy.

Results

Observation of Cultured Adherent Cells Derived From Bone Marrow Populations

Light Microscopy

The bone marrow adherent cells were similar morphologically in both infected and noninfected guinea pig cells (Table 1). During the first 3 days of cultivation, macrophages were predominant. The guinea pig macrophages were pleomorphic, ranging from polygonal to stellate cells exhibiting ruffled membranes and pseudopods. Their nucleus was single or multiple and contained a prominent nucleolus. Spontaneous phagocytosis of debris, crystals, or dead cells was invariably seen, the latter comprised mostly of polymorphonuclear leukocytes and rare erythrocytes. No contact or confluency was noted among macrophages. After 4 to 7 days of cultivation, increasing numbers of spindle-shaped cells appeared and grew to confluency in compact islands. These fibroblastic cells had a smoother contour without pseudopods, lighter basophilic cytoplasm, and a vesicular nucleus with several chromocenters.

Phagocytosis of latex was present in the macrophages and absent in the fibroblasts. Likewise, dark brown coarse granularity was present in macro-

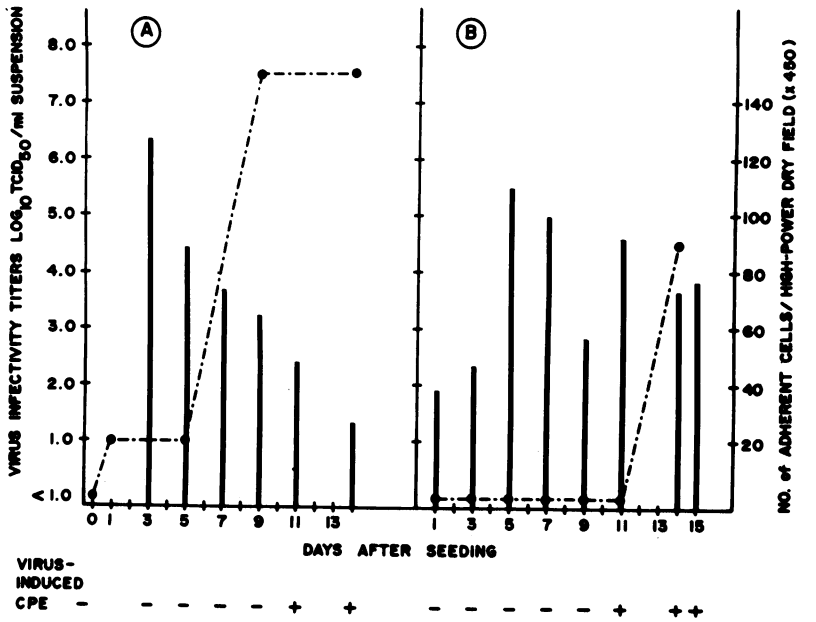
phages and absent in fibroblasts when the preparations were processed for NEE reaction. Frequently, spontaneous debris phagocytosis and positive NEE reaction occurred in the same cell. When virus-induced CPE appeared in the marrow cultures from latently infected animals, usually 10 days or longer after seeding, cell degeneration and inclusion bodies were observed in both macrophages and fibroblasts (Figure 1).

Electron Microscopy

Adherent cells obtained from uninfected and infected guinea pigs exhibited similar morphology by electron microscopy prior to the virus-induced changes which occurred only in the infected cells. Among the cultured cells, the nonconfluent polygonal cells possessed irregular contours with coarse and delicate pseudopodic projections. Their nucleus or nuclei had dense, marginated nuclear chromatin. The cytoplasm contained numerous mitochrondria and prominent smooth endoplasmic reticulum. A well-developed Golgi apparatus was often found in the paranuclear area, close to the concave side of the nucleus. Also present near the concave side were variable amounts of lysosomal vesicles and digestive vacuoles containing cell debris, both amorphous and structurally organized (Figure 2A). The phagosomes contained incomplete or complete nucleated cells in different degrees of disintegration, usually beyond recognition.

The confluent spindle cells had regular outline and lacked cytoplasmic projections (Figure 2B). Their nucleus showed loose chromatin only thinly distributed along the nuclear membrane. Several granular chromocenters were present. The cytoplasm contained abundant rough endoplasmic reticulum and few mitochondria.

Herpesvirus virions were found in both cell types. In the polygonal phagocytic cells, enlarged nuclei containing numerous virions were seen (Figure 3). These herpes-like particles were occasionally seen in nuclear "packages" in which they appeared to be enveloped. Rare conglomerates of virus particles were seen in the cytoplasm, although isolated particles were found. Most of the enveloped particles were located outside the cytoplasmic membrane. Particles resembling herpes-like virus were also observed in the phagosomes located in the cytoplasm. Whether the latter particles were actually viral in nature was not determined. The fibroblast cells exhibited similar evidence of herpesvirus infection but contained no phagosomes.



TEXT-FIGURE 1—Expression of latent GPHLV in cultured adherent bone marrow cells. Virus infectivity titers, *broken lines*; cell counts, *solid lines*. A—Prolonged adherent cells. B—24-hour adherent cells.

Expression of GPHLV in Cultured Adherent Cells Derived From Bone Marrow of Latently Infected Guinea Pigs

Two types of marrow cultures were used. In the first group of experiments, whole marrow cell populations were allowed to attach and grow out after initial seeding of approximately 10^7 cells/ml obtained from infected guinea pigs. Serial daily samples of the adherent cells, after removing the unattached cells, were assayed for virus infectivity titers. The virus was first detected on Day 9 after seeding, and viral inclusion bodies were detected on Day 11. The final virus yield was 7.5 log $TCID_{50}$/ml of packed cultured marrow adherent cells (Text-figure 1A). In the second group of experiments, whole marrow cells were allowed to adhere for 24 hours; unattached cells were removed by washing vigorously three times with HBSS and were replaced with medium RPMI 1640. Serial samples of the growing adherent cells were assayed for virus infectivity titers. Significant virus titers were detected in the cultures harvested on Day 14, although virus-induced nuclear inclusions were observed on Days 11, 14, and 15. The final virus yield was 4.5 log $TCID_{50}$/ml of packed cultured cells (Text-figure 1B).

Table 2—Detection of GPHLV in Cells Derived From Bone Marrow of Latently Infected Guinea Pigs After Different Intervals of Adherence

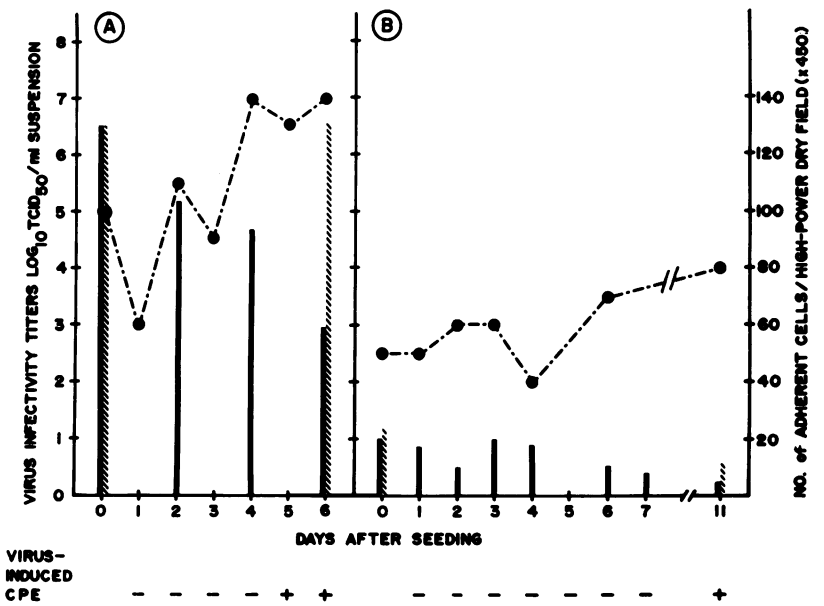
Original cell concentration ($\times 10^6$/ml)	Initial virus-infected titers log $TCID_{50}$/0.1 ml of packed cells	No. of samples that showed virus CPE/No. of samples tested		First appearance of CPE in reverse cocultivated cultures (days)	
		30*	120	30	120
2	2.5	ND	0/11†	ND	None
10	3.0	ND	2/3	ND	16
100	2.0	2/12	3/6	7	7
100	2.0	2/4	1/3	5	5

ND = Not done.
* Adherence time, 30 minutes and 120 minutes
† Cultures were held for 4 weeks.

In both groups of experiments, parallel coverslips were stained with hematoxylin and eosin for counting relative density of cells per high-power microscopic field (× 450). In all instances, the initial donor bone marrow cells showed a virus infectivity titer of 3.0 log $TCID_{50}$/ml of packed cells by cocultivation. However, the final virus yields from these cultures varied from 4.5 to 7.5 log $TCID_{50}$. It appeared that when the initial cell concentrations were high, the virus yields were also high (Text-figure 1A). Apparently some of the marrow cells carrying virus in the initial marrow cell suspension were removed during the washings 24 hours after seeding (Text-figure 1B).

Detection of GPHLV in "Early" Adherent Bone Marrow Cells From Latently Infected Guinea Pigs

Bone marrow cell suspensions containing 2, 10, or 100 × 10^6 cells/ml were allowed to attach to the glass surface at 37 C in a CO_2 incubator for 30 and 120 minutes, after which the nonadherent cells were removed by washing thoroughly three times with HBSS. The adherent cells were then overlaid with GPEF cell suspension to obtain a reverse cocultivation system. Virus-induced CPE in the overlaid fibroblasts was recorded. The results are shown in Table 2. Typical virus-induced CPE appeared in the overlaid cultures that were initially seeded with 10 × 10^6 or greater. The number of samples that showed CPE varied from experiment to experiment. Control GPEF cell cultures did not show CPE at any time. When the donor marrow cell concentration was high, ie, 10^8 cells/ml, virus activity appeared sooner, 5 to 7 days, even in cultures of cells that were allowed only 30 minutes for attachment. In lesser cell concentrations, the appearance of virus-induced CPE was delayed. When the donor marrow cell concentration at seeding was low or less than 2 × 10^6 cells/ml, virus



TEXT-FIGURE 2—Replication of GPHLV in cultured bone marrow adherent cells. Virus infectivity titer, *broken lines*; infected cell count, *solid bar graphs*; uninfected cell count, *slashed bar graphs*. A—Four-day-old cultures. B—One-day-old cultures.

activity could not be detected, even when the cultures were held for 4 weeks.

GPHLV Infection in Cultured Adherent Cells Derived From Bone Marrow of Noninfected Guinea Pigs

Bone marrow cells obtained from noninfected Hartley guinea pigs were seeded into Leighton tubes. After 1 to 4 days they were washed thoroughly three times with HBSS and were inoculated with 0.1 ml of LK40 stock virus suspension containing 10^5 $TCID_{50}$. After 2 hours of adsorption, nonadsorbed virus was removed and replaced with fresh RPMI 1640. Serial samples were assayed for virus infectivity titers after the cell-containing tubes were frozen and thawed three times. Parallel coverslip cultures were fixed for cell counts. In two separate experiments, using 1- and 4-day-old marrow cell cultures, increasing virus yields were obtained following *in vitro* infection with GPHLV (Text-figure 2). Virus inclusions were observed in adherent cells at Days 5 and 11 of infection, while the final virus titers varied from 6.5 to 4.0 log $TCID_{50}$/0.1 ml of packed cultured cells, in the 4- and 1-day-old cultures, respectively. Unlike control uninfected adherent cells, the infected cultures showed a steady decrement of cell density.

Discussion

The present study showed that following cultivation of bone marrow cells derived from infected guinea pigs, virus yields were higher in cultures with prolonged attachment of adherent cells than in those cell cultures permitted only 24 hours for adherence. Expression of virus increased progressively in both cases. Generally, virus infectivity titers appeared prior to evidence of virus-induced CPE or intranuclear inclusions. Identification of the cell types by light and electron microscopy revealed that most of the adherent cells showed phagocytosis of dead cells, debris and latex particles, and positive NEE reaction, thus fulfilling the criteria for macrophages.[8-14] However, increased numbers of fibroblasts were also noted at the time infectious virus was detected. (See Table 1, last column.) Herpesvirus intranuclear inclusions were found in both cell types (Figure 1). It was not possible to identify the specific cell type carrying the herpesvirus genome, although virus particles were seen in macrophages, which were characterized by the presence of phagosomes and the nucleocytoplasmic features previously described.

Attempts to obtain infectious centers in adherent marrow cells cultured with GPEF under either methyl cellulose or agar overlay were unsuccessful since these cultures could not be maintained for sufficiently long periods to permit expression of latent virus.

In an earlier study of GPHLV expression in the cells in organ cultures of spleen and thymus tissue derived from GPHLV infected guinea pigs, it was found that in the presence of specific antibody, GPHLV was not expressed in individual cells, as indicated by the absence of virus-induced inclusions.[4] A similar experiment could potentially have provided valuable information in the present study. However, more effort will be necessary to develop a procedure by which the cells can be maintained in good condition for longer periods to permit the expression of the latent GPHLV.

It was not certain whether the herpesvirus observed in the macrophages was an integral part of the cell genome or whether it occurred as a result of the macrophage taking the virus secondarily from the extracellular medium or from an infected cell which had been phagocytosed. The contagion by phagocytosis of degenerated virus-infected cells is an attractive interpretation in view of the marked phagocytic activity exhibited by macrophages *in vitro*. In one instance, as shown in Figure 3B, virus-like particles were observed within an unidentified cell engulfed by a macrophage undergoing productive viral infection. Whether these particles were viral could not be determined. Such a phenomenon, however, was not commonly observed in several other experiments. Whether a princi-

pal, secondary, or concomitant mechanism was responsible for acquisition of the virus by macrophages remains to be elucidated.

It was also demonstrated in these experiments that the adherent cells of the macrophage-enriched population were susceptible to *in vitro* infection with GPHLV, although the increase in virus titers was higher in the 4-day-old cultures than in the 1-day-old cultures. This was probably due to the small number of cells in the 1-day-old cultures at the time of infection. Since a 4-day-old culture may have contained a mixed population (Table 1), it was not certain whether the fibroblasts and macrophages had the same infection rate. Under light and electron microscopy, however, virus-induced intranuclear inclusions and virions were apparent in both cell types in a fashion similar to that seen in marrow cultures derived from latently infected guinea pigs.

Experiments with the "early" adherent bone marrow cells, ie, 30 minutes or 120 minutes of attachment, did show the presence of GPHLV under the following conditions: a) a concentrated cell population, ie, 10^7 to 10^8 cells/ml for initial attachment and b) reverse cocultivation with susceptible cells or GPEF. The higher the initial cell concentration used for attachment, the faster the apprearance of virus-induced CPE occurred (Table 2). Despite previous reports that highly purified macrophage populations can be obtained following brief periods of attachment of Ficoll-Hypaque–separated peripheral blood cells,[15] the general impression from the present study was that the cells in these bone marrow preparations were more varied, perhaps due to the mixed population including promonocytes, monocytes, and macrophages. Thus, cultured cells derived from the bone marrow may be of a similar mixed nature.

Mononuclear phagocytes have been implicated in several productive, acutely developed viral infections.[16-24] These experiments have been carried out in an attempt to explain the removal, *in vivo* and *in vitro*, of infectious virus particles by these mononuclear cells, the reasons for their success or failure in opposing a containing barrier to dissemination, and the virus-related cellular reactions in which cells of this system participate. However, less effort has been made to elucidate the possible role of mononuclear phagocytes in the latency of viruses involved in persistent infections. Attempts were made in the present study to examine the *in vitro* susceptibility to GPHLV of a macrophage-enriched population derived from normal bone marrow adherent cells and to determine the degree of *in vivo* persistence of the virus in cells derived from GPHLV infected guinea pigs. Although GPHLV was found capable of replicating and persisting in macrophage-enriched populations, the importance of the mononuclear phagocytes in the establishment of latent GPHLV infection *in vivo* remains an open question.

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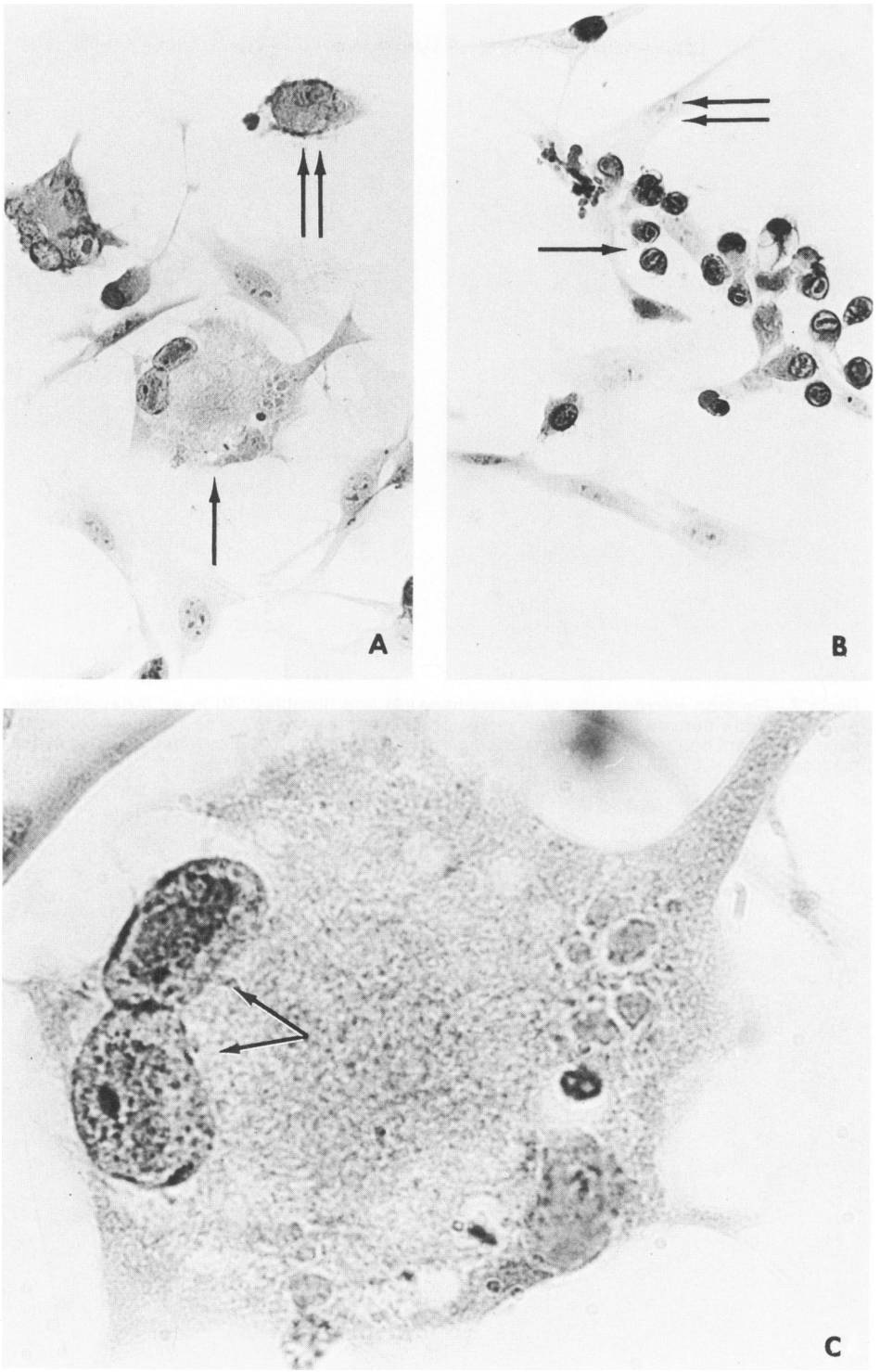
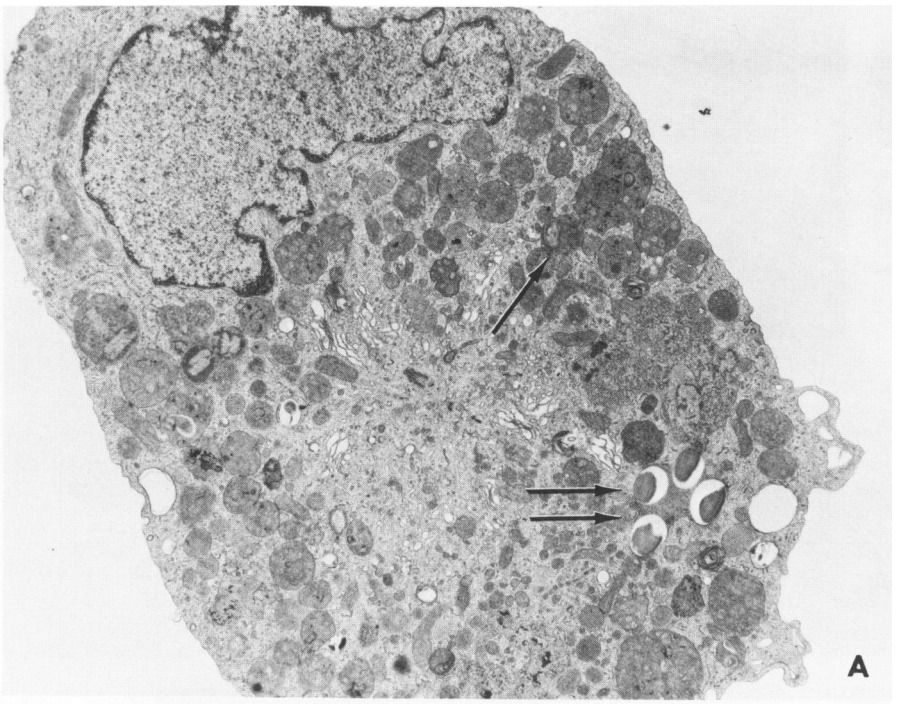


Figure 1—A 14-day-old culture of bone marrow adherent cells derived from a latently infected guinea pig. **A**—Macrophages with (*single arrow*) and without (*double arrow*) virus-induced intranuclear inclusions. **B**—Fibroblasts with (*single arrow*) and without (*double arrow*) virus-induced intranuclear inclusions. **C**—Detail from **A**, showing a multinucleated macrophage bearing several intranuclear inclusions (*arrows*). Note cytoplasmic projections and phagosomes containing engulfed debris. (H&E; **A**, × 450; **B**, × 450; **C**, × 1000)

Figure 2—Electron micrographs of macrophage (A) and fibroblast (B) in an 8-day-old bone marrow culture derived from a latently infected guinea pig prior to the appearance of virions. Note prominent phagocytosis of debris (*single arrow*) and latex particles (*double arrow*) by the macrophage. (× 6750)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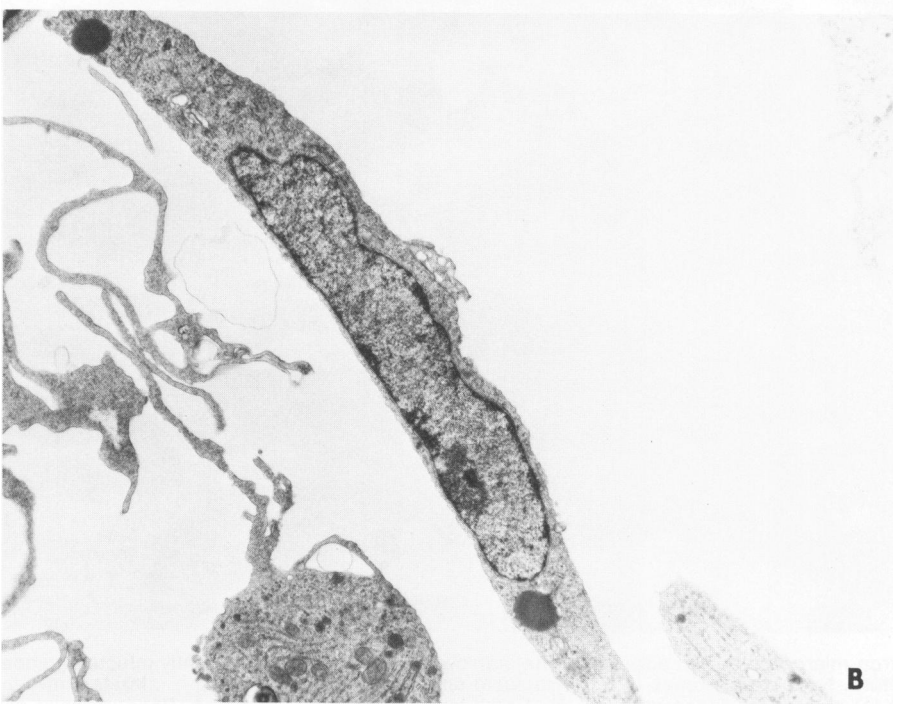
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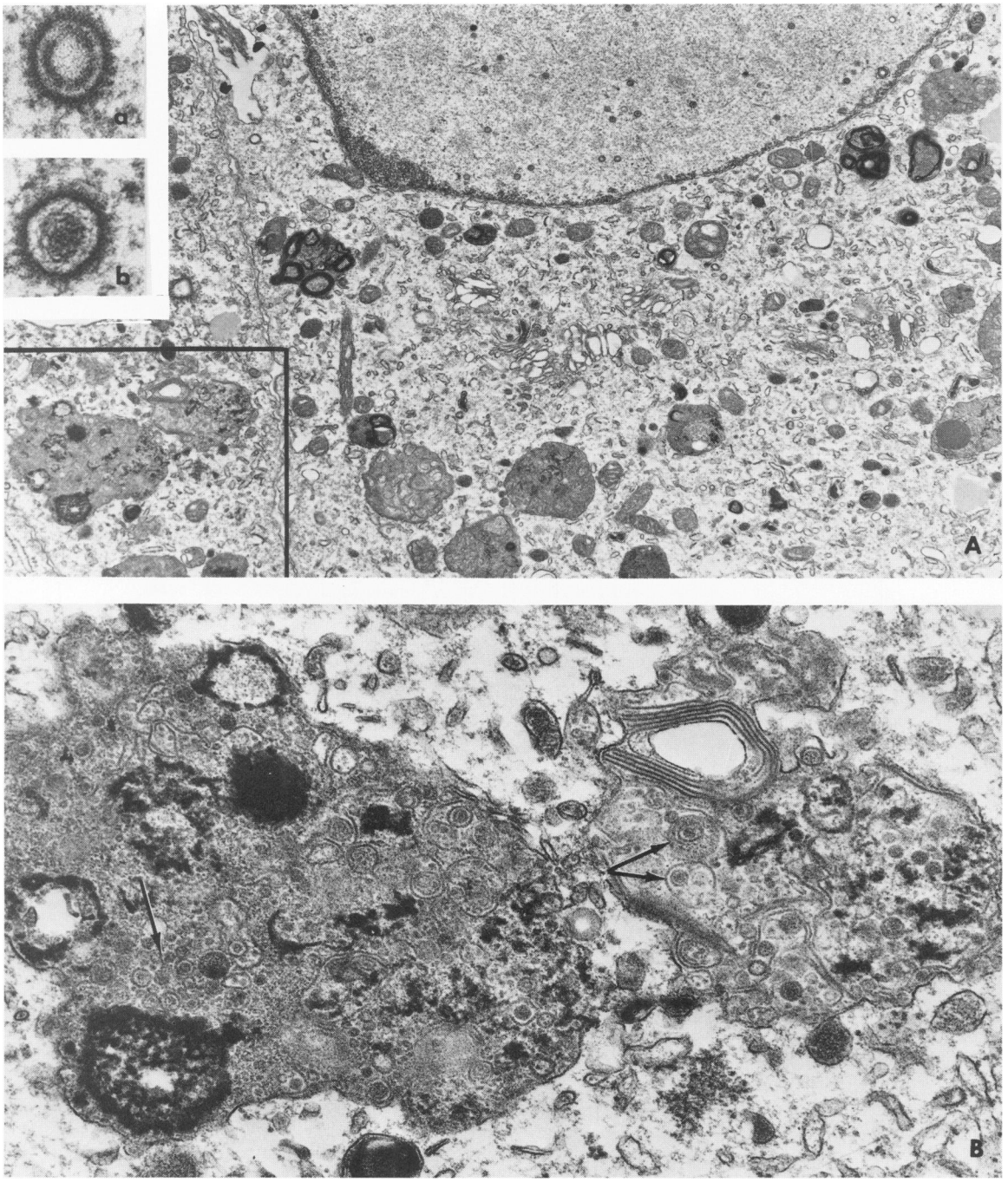

Figure 3—Electron micrograph of an adherent bone marrow macrophage from a latently infected guinea pig. **A**—15-day-old culture. Note phagosomes in the cytoplasm and virions in the nucleus. **Insets**—Intranuclear herpes virions without (a) or with (b) dense core. **B**—Detail from the marked area of **A**. Phagocytosed cells exhibiting advanced degeneration. Note numerous virus-like particles in the phagosomes (*arrows*). (**A**, × 9360; **Insets**, × 132,000; **B**, × 38,400)