In Vitro Cell-Virus Interactions Associated with *Bovine herpesvirus-1* and Peripheral Bovine Blood Cellular Components

By

PHILIP NJERU NYAGA
B.V.M. (University of Nairobi) 1973
M.P.V.M. (University of California, Davis) 1975

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Comparative Pathology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

Committee in Charge

Deposited in the University Library 1975
Date *Librarian*

DEDICATION

This manuscript is dedicated to my parents, Benjamin and Margery Njeru wa Nyaga; and further as a tribute to Professor, Dr. Delbert G. McKercher's work on viruses of domestic animals.

ACKNOWLEDGEMENTS

I wish to thank Dr. McKercher for offering freely of his time, academic advice and constant encouragement throughout the training period in which this work was done. His assistance and that of Drs. J. S. Manning and B. I. Osburn in various aspects of the project and in writing the dissertation are gratefully appreciated.

Furthermore, I am grateful for educative discussions relevant to this work with Drs. J. W. Osebold, C. Stormont, R. Yamamoto, A. M. Buchanan, and Y. C. Zee; C. E. Franti and A. D. Wiggins. The kindly help on administrative matters rendered by Drs. E. L. Biberstein, C. Stormont, D. E. Jasper and R. A. Bankowski are joyfully acknowledged.

Moreover, I thank Mrs. Claire Drew, Miss E. M. Wada and Dr. Hiroshi. Ito for assistance with laboratory work; and also Dr. Jack Pangborn, Miss Janie R. Woods and Mr. Edward E. Callaway for invaluable help with electron microscopy. To my fellow graduate student, David Labarre, I extend special regards for the time we spent together discussing this project.

Finally, I wish to acknowledge, with thanks, the leave of absence from the University of Nairobi (Kenya, East Africa) and the financial support from the Rockefeller Foundation.

CONTENTS

-- TITLE PAGE . . . . . (i)
-- DEDICATION . . . . . (ii)
-- ACKNOWLEDGEMENTS . . . . . (iii)

I. INTRODUCTION 1

II. MATERIALS AND METHODS

A. MISCELLANEOUS ITEMS 4
(i) Media 4
(ii) Indicator Cells 4
(iii) Stock Virus 5
(iv) Virus assay in microtiter system 5
(v) Delivery of virus 6

B. SEPARATION & MAINTENANCE IN CULTURE OF BLOOD CELLS 7

C. ADSORPTION
(i) In whole blood 9
(ii) Separated cells
a) Leukocytes and red blood cells 10
b) Mononuclear cells 11
c) Nonadherent mononuclear cells 11
d) Polymorphonuclear cells 11

D. REPLICATION
(i) Whole blood 11
(ii) Red blood cells 12
(iii) Leukocytes 12
a) Adherent mononuclear and Bovine embryonic lung cells 13
b) Nonadherent mononuclear cells 14
c) Polymorphonuclear cells 15

E. MORPHOGENESIS
(i) Adherent mononuclear and Bovine embryonic lung cells . . 15
(ii) Nonadherent mononuclear cells 17
(iii) Polymorphonuclear cells 17

III. RESULTS

A. CELL SEPARATION 19

B. ADSORPTION
(i) Whole blood 20
(ii) Separated cells 20

C. REPLICATION
(i) Whole blood 20
(ii) Separated cells
a) Bovine embryonic and adherent mononuclear cells . . . 21
b) Nonadherent mononuclear cells 22
c) Polymorphonuclear cells 23

D. MORPHOGENESIS
(i) Bovine embryonic lung cells 24
(ii) Adherent mononuclear cells 24
(iii) Nonadherent mononuclear cells 25
(iv) Polymorphonuclear cells 25

IV. DISCUSSION 27

V. SUMMARY AND CONCLUSIONS 34

VI. BIBLIOGRAPHY 35

VII. TABLES 44

VIII. FIGURES 45

IX. ABSTRACT 105

X. APPENDIX: STATISTICAL ANALYSES 108

A. COMPUTATION OF 50 PERCENT END POINTS 109

B. THE GEOMETRIC MEAN 111

C. EVALUATION OF SIGNIFICANCE 112

IN-VITRO CELL-VIRUS INTERACTIONS ASSOCIATED WITH BOVINE HERPESVIRUS-I AND THE PERIPHERAL BOVINE BLOOD CELLULAR COMPONENTS

Bovine herpesvirus-1 {(BHV-1); 103} commonly known as the infectious bovine rhinotracheitis (IBR) virus, is a unique disease producing agent of cattle throughout the world. In retrospect it is now realized that for many years this virus caused a genital disease of cattle known as coital vesicular exanthema or colloquially as "Blaschenausschlag" in central Europe (110). Subsequently the virus was isolated in North America from cattle affected with a respiratory tract syndrome which became known as infectious bovine rhinotracheitis (IBR) and eventually shown to be associated etiologically with a diversity of clinical disease syndromes in cattle (64). These include rhinotracheitis (55), conjunctivitis (1), meningoencephalitis (7, 32), vaginitis (65, 68) and abortion (66). In some instances, the virus has been associated with keratoconjunctivitis in cattle (45) whereas in others it has been isolated from cattle with infectious infertility in South Africa (61). Evidence from experimental infection of calves also implicates the virus as the cause of an enteric disease syndrome (6, 16, 35, 36).

The diverse nature of the infections caused by this virus indicates that it adapts rapidly to a wide range of host tissues. This also implies that the virus enters the body through several independent routes and is subsequently distributed in the body by a number of means.

Early studies showed that respiratory syndrome was contracted through droplet infection. Conjunctivitis has been reproduced experimentally by direct instillation of viral culture into the palpebral sac and by nasal exposure. However, the natural infection is thought to arise following the invasion of the conjunctival tissues via the lacrimal ducts.

On the other hand, the genital syndromes known as infectious pultular vulvovaginitis (IPV), and infectious balanoposthitis (IPB) have been reproduced experimentally by applying the virus to the vaginal or penile mucosa (96). Natural transmission is by venereal means.

The enteric infection by BHV-1 probably results from the ingesting of virus-laden secretions from the nasal passages during the acute phase of the respiratory infection. However, the manner in which the virus reaches the placental tissues or the brain, subsequent to vaccination or to an acute attack of IBR was unclear. It was obvious that it had to pass through the blood stream in order to reach these target organs. However, repeated failures to recover virus from whole blood was puzzling. Subsequently, however, it was isolated fairly consistently from the buffy coat cells of cattle exposed to the virus via the nasal route, and from the fetuses aborted by these animals (66, 67). Similar observations had been made in cases of herpesviral encephalitis in calves in Australia (32). Thus, an association between Bovine herpesvirus-1 and the white blood cells components of the blood was established.

The nature of this association was the subject of considerable speculation. In view of the fact that a low serum antibody titer to the virus prevented its passage through the blood stream suggested that the virus was located at a site, or sites, where it came into direct contact with specific antibody. The most logical site, therefore, would be the cell surfaces. Another possibility was that the virus underwent replication in the blood stream but in so few cells that its concentration never reached the threshold level required for its isolation. It is also possible that the rate of viral replication in the blood cells is very low, accompanied by a minimal viral release.

The object of the present study was to determine the validity, or lack of same, of the hypotheses advanced. Since this study was an *in vitro* model, its scope was restricted to a determination of 1) the types of white blood cells involved and 2) the interactions between these cells and the virus. Although of an *in vitro* nature, it was felt that it would serve as a reasonable correlate of the *in vivo* cell - virus interactions in the animal body.

Initial studies were done to determine whether cells in whole blood could adsorb and support the replication of BHV-1. Later, the cells that could adsorb the virus and act as its host cells were identified. In order to do this, pure preparations of the various cellular components of whole blood were obtained. Such fractionation of the blood was possible utilizing the variations in size, density and the biological behavior of the cellular elements as a basis for their physical separation.

Normally, the rates of sedimentation of these cells in a gravitational field would be more related to size than to cellular density. However, by bringing the density of the suspending medium close to that of the cells to be separated, the sedimentation rates relate more to the cellular densities. Such a density was provided by a Ficoll-Hypaque gradient to be described below.

When diluted bovine blood was centrifuged over this gradient, a distinct band of mononuclear cells was obtained. The granulocytes sedimented together with the red cells and were recovered later from these sediments. The lymphocytes were then recovered from the mononuclear cell band.

The manner in which the pure cell suspensions were obtained is described below.

II. MATERIALS AND METHODS

A. MISCELLANEOUS ITEMS

(i) Media:

Saline-A and Trypsin-Versene are described below in the relevant appropriate sections. Eagles Minimum Essential Medium (MEM) was used as the basal medium throughout. A growth medium consisted of MEM plus antibiotics and a high fetal calf serum (FCS) supplement; while maintenance medium contained less concentration of FCS.

(ii) Indicator cell cultures:

The cell cultures used for the virus assay were secondary monolayer cultures of BEL cells. They were used routinely at the 2nd to the 8th passage levels in microtiter plates (MT).*a Cell suspensions to be used in the MT plates were prepared from the secondary monolayer cultures grown in 16-oz. glass bottles as follows: The spent medium was removed and collected in 40 ml centrifuge tubes. The cell monolayers were rinsed twice with saline-A (Sal-A)*b and overlaid with enough prewarmed (37°C) Sal-A trypsin-versene (Sal-A-T-V)*c to cover the monolayers. The Sal-T-V was decanted and the bottles held for 5 to 10 minutes in an air incubator. The bottles were removed from the incubator and three mls of Sal-A added to each in order to resuspend the detached cells. All cell suspensions were pooled and mixed with an equal volume of homologous spent medium. They were centrifuged at 200xg for 10 minutes. The pelleted cells were resuspended at

---

*a Linbro Chemical Co., New Haven, Conn. or Cooke Engineering, Alexandria, Virginia.

*b Consisting of 8.0 grams NaCl, 0.4 gms KCl, 1.0 gm glucose, 0.35 gm $NaHCO_3$ per ml; 250 I.U. penicillin and 250 mg streptomycin per ml.

*c Saline A + Trypsin (0.05%) + Versene (Disodium Ethylenedinitrinotetracetate 0.02%). Trypsin and versen were obtained from General Biochemicals and J. T. Baker Co., Phillipsburg, N. J., respectively.

a concentration of 200,000 cells per ml in freshly prepared growth medium*d and dispensed in 0.05 ml amounts per MT plate well. The MT plates were sealed and incubated in an atmosphere of 5% carbon dioxide (CO_2, v/v). Cells were ready for use within 24 to 48 hours. They were rinsed once with Sal-A before use.

(iii) Stock virus cultures:

The virus used in the present study was the Los Angeles strain (IBR-LA) of BHV-1, previously isolated in this laboratory. It was obtained at the second passage in bovine embryo kidney (BEK) cells and plaque purified three times. A single plaque was picked from the final plaquing and further passed five times to increase the titer. The stock virus was tested for mycoplasma contamination and against standard IBR-LA antiserum. It was shown to be free of mycoplasm and to be completely neutralized by IBR-LA antiserum. The stock virus was stored at -80 C in 2 ml volumes/vial.

(iv) Virus Assay in Microtiter Plates:

All viral titrations were carried out in an identical manner in MT plates. One general description will suffice to cover the manner in which the viral content of the stock virus and the samples from the adsorption and growth curve studies were assayed. To titrate stock virus, serial 10 fold dilutions were made in Dulbecco's phosphate buffer (DPB). Samples from the adsorption and growth curve studies were carried out in the same buffer. Starting with a dilution of 1:10, samples were serially diluted in two-fold steps to the desired endpoint, depending on the experiment and the technical limitations of the microtiter equipment. The end points were either 1:1280 or 1:20480 depending on whether the plates were used width- or length-wise.

---

*d Eagle's Minimum Essential Medium (MEM), fortified with 10% (v/v) FCS, and 100 I.V. Penicillin and 100 mg streptomycin per ml.

Whenever samples from whole blood or plasma were to be diluted, calcium-magnesium free DPB was used as diluent to avoid clotting of the plasma proteins which would hinder the free diffusion of virus during the titrations.

If the samples were considered to contain virus in excess of four logarithmic (log) units per ml, an initial dilution of 1:10 was made, followed by a second of 1:640 and further two-fold serial dilutions to the desired endpoint. All dilutions were made in 13 x 100 mm disposable culture tubes*e and later transferred to the MT plates.

(v) Delivering Diluted Virus onto Microtiter Cultures:

Disposable plastic microdropper pipettes calibrated to deliver accurately 0.025 ml volumes per drop were used to transfer viral cultures from the dilution tubes to the MT plates. A fresh pipette was used for each sample. Starting with the highest dilution, a drop of each sample was transferred to each of five wells. The lowest dilution of virus was delivered last. Sufficient control cell cultures were included for each set of 4 MT plates. Microplates containing the diluted viral suspensions were covered with loose fitting plastic lids, and held for four hours at 37 C in a humidified incubator containing 5% CO_2.*f At this time the MT plates were removed from the incubator and 0.05 ml of maintenance medium containing 5% FCS was added to each well. The MT plates were then sealed with plastic sealing tape,*g and returned to the incubator. After 48 to 72 hours, the MT plates were checked for cytopathic effect (CPE).

Virus content was calculated by the method of Spearman-Kaerber (46) and expressed as median tissue culture infectious doses ($TCID_{50}$) per 0.025 ml.

---

*e Kimble, Division of Owens-Illinois

*f National Appliances Co., Portland, Oregon, Model 3221, Serial 6J65

*g 3M Company, St. Paul, Minnesota 55101

Titers were converted to $TCID_{50}$ per ml by adding 1.6[*h] to the log $TCID_{50}$ per 0.025 ml. Titers per tube or per flask were computed by multiplying the titer per ml by the volume before sampling for the corresponding samples in each experiment.

B. SEPARATION OF CELLULAR ELEMENTS OF BOVINE BLOOD AND THEIR MAINTENANCE IN CELL CULTURE

Part of the apparatus used is shown in figure (2), while the separatio procedure is summarized in figure (1). The detailed procedure is as follows:

Bovine blood was drawn by venipuncture into 10 ml vacutainer tubes containing tripotassium ethylenediaminetetraacetate (EDTA); 1.5 mg per ml of blood, and taken to the laboratory within 30 minutes of collection. The blood from each of four tubes was immediately dispensed into a 125 ml serum bottle and diluted 1:3 by adding approximately 80 ml of calcium-magnesium-free DPB to each bottle. The diluted blood (18.5 ml/tube) was layered carefully over the Ficoll-Hypaque gradient solution (8.0 ml/tube).[*i] A distinct interface was formed between the blood and the gradient solution. However, in order to facilitate the smooth delivery of the diluted blood onto the gradient surface without disturbing the interface, the walls of each tube had been coated with a thin layer of the gradient solution. A small amount of blood was then run gently down the moist tube walls in a smooth broad band, reaching the gradient surface as a broad fluid front. The rest of the blood was added until each tube was filled to the shoulders

*h : Explained in the appendix.

*i : The gradient solution was made by mixing 5mls of 9% Ficoll with 3 mls of 33% (v/v) Hypaque immediately before use. The solutions had been sterilised by membrane filtration (Millipore Corporation, Bedford, Massachusetts 01730; #0.22 mu); Ficoll: Pharmacia Fine Chemicals, Inc., 800 Centenial Avenue, Piscataway, N.J.; Hypaque: Winthrop Laboratories, 160 Scott Drive, Menlo Park, California 94025.

The blood was centrifuged at 100 x g for 20 minutes. The plasma fraction (layer 1) was aspirated to a point 5 mm above the distinct white band of mononuclear (MN) cells (layer 2) which had formed at the original blood-gradient interface. The MN-rich fraction was aspirated to a point just 2 mm below the interface and the polymorphonuclear (PMN) cell-rich fraction (layer 3) was then aspirated, leaving the erythrocyte-PMN fraction in each tube. The PMN cells in the sediment (layer 4) were recovered by lysing the erythrocytes with distilled water, followed by reconstitution to tonicity with phosphate buffered saline. The resultant PMN cell suspensions were added to those of layer 3. The MN and the PMN cells from layer 3 were freed of contaminating red cells in the same manner. One-tenth ml fractions of each leukocyte preparation was then mixed with 0.9 ml of 0.05% trypan blue vital stain and examined in the Improved Neubauer hemacytometer*j for viability and cell concentration enumerations.

To obtain glass-adherent and non-adherent MN cells, the MN cell preparations were washed twice in DPB and resuspended in growth medium, fortified with 20% FCS and antibiotics (penicillin 100 I.U., streptomycin 100 mg and 75 mcg gentamicin). The cells were passed through a series of transfers in 16-oz. glass bottles, and incubated for 3 hours before each transfer. The cells that remained in suspension were centrifuged at 150 x g for 15 minutes. The cell pellets were resuspended at appropriate concentrations for the different assays. Adherent cells were recovered from the glass bottles by elution, using several changes of buffered trypsin-versene (0.25% trypsin and 0.02% versene). The adherent cell suspensions were washed once before reculturing at appropriate concentrations for the different experiments.

---

*j Bright Line, Hemocytometer, American Optical Company, Scientific Instrument Division. Buffalo, N. Y. 14215.

For the recovery of erythrocytes, the sediments (layer 4) from several tubes were pooled, diluted 1:3 and recentrifuged at 1200 x g for 20 minutes. Any leukocytes which collected at the fluid-red blood cell (RBC) interface were removed and discarded. The red cells were obtained by introducing the tip of a pipette to the bottom of the red cell sediment and aspirating a substantial volume of the latter. These cells were washed twice and resuspended to the appropriate concentrations for different assays.

To obtain a preparation of unfractionated leukocytes, whole bovine blood in an anticoagulant was centrifuged at 1200 x g for 15 minutes. The plasma was withdrawn to a point approximately 10 mm above the surface of the white band of leukocytes and discarded. The leukocytic fraction was aspirated, together with approximately a 20-25 mm layer of the RBC sediment and pooled in a centrifuge tube. The RBC cells were lysed with distilled deionized water followed by reconstitution to tonicity within 30 seconds.

The lysates were centrifuged at 150 x g for 15 minutes. The resultant cells were washed twice in calcium-magnesium free DPB, and tested for viability by trypan blue staining. The cell suspensions were concentrated or diluted in Eagle's MEM fortified with 20% by volume of FCS and antibiotics.

C. ADSORPTION OF BOVINE HERPESVIRUS-I BY CELLULAR ELEMENTS OF BOVINE BLOOD

(i) Adsorption by cells in whole blood:

To determine whether bovine herpesvirus-1 would be adsorbed by cells in whole bovine blood, 90 ml of such blood and 90 ml of freshly obtained normal bovine plasma were divided into six portions, each containing 13.5 ml; the remaining blood and plasma were discarded. Each portion was placed in a screw-capped tube (20 x 125 mm, Pyrex) and held at 4 C. Stock virus culture was diluted in DPB so as to provide $10^{6.78}$ and $10^{5.78}$ median

tissue culture infectious doses ($TCID_{50}$), per ml of diluent. One and a half ml of each viral preparation were mixed with 13.5 ml of either the blood or the plasma portions contained in each of any of the three tubes randomly chosen from the stock pool of tubes as described above. Thus, the virus culture was diluted ten-fold resulting in two dose levels, namely the high ($10^{5.78}$ $TCID_{50}$ per ml) and the low ($10^{4.78}$ $TCID_{50}$ per ml). All tubes were incubated at 4 C. Two ml samples were withdrawn from each tube at 1, 6, 12, 18 and 24 hours. At each sampling, the tube contents were thoroughly mixed prior to obtaining the sample. Supernatant fractions were obtained after centrifuging the samples at 150 xg for 15 minutes. They were stored at -80 C. When sampling was completed, the samples were assayed for viral content as described above. Bovine plasma was used in this experiment and in the subsequent adsorption experiments as a control for nonspecific viral inactivation.

(ii) Adsorption by Separated Blood Cells:

(a) Leukocytes and Red Blood Cells:

In order to determine the cells in whole blood which adsorb the virus, the blood was initially fractionated into the leukocytic and red cell components. Platelets were not tested for adsorption capacity. Later the leukocytes were separated into their respective cellular components. A suspension of leukocytes containing 2.0×10^6 cells per ml and of red cells containing 8.0×10^9 cells per ml were prepared. Each cell type was distributed into a series of 6 tubes, each containing 13.5 ml of either cell type. Each series was divided into 2 groups of three tubes each. One and one half ml of viral culture containing $10^{6.78}$ per ml was added to three of the tubes in each series, and the same volume of virus containing $10^{5.7}$ $TCID_{50}$ per ml was added to the remaining 3 tubes of each series. A two ml sample was withdrawn from each tube at the same time intervals as for whole

blood. Samples were centrifuged at 150x g for 15 minutes and fractions from the corresponding supernatants were removed and stored at -80 C. They were subsequently tested for viral content as described above.

(b) Mononuclear Cells:

A suspension of MN cells containing 9.72×10^6 cells per ml was distributed into 6 tubes, each containing 13.5 ml. The tubes were divided into two groups of three tubes each. To each tube in the one group, 1.5 ml of a BHV-1 culture containing $10^{6.78}$ $TCID_{50}$ was added. The remaining cell suspensions each received 1.5 ml of the viral culture. All tubes were incubated at 4 C and 2 ml samples were withdrawn from each tube. After centrifugation, supernatants were recovered for viral content assay as described above.

(c) Nonadherent Mononuclear Cells:

These cells were tested for adsorption capacity in the same manner as described above for the total mononuclear cell component.

(d) Polymorphonuclear Cells:

These cells were tested for viral adsorption indirectly by electron microscopic examination for evidence of viral uptake and replication. Since adsorption is known to be a necessary prelude to the infection of any cell by any virus, it was assumed that evidence of viral replication e.g. viral particles in the nuclei of cells, would indicate previous adsorption of the virus to such cells.

D. REPLICATION OF BOVINE HERPESVIRUS-I IN WHOLE BOVINE BLOOD AND ITS CELLULAR COMPONENTS

(i) Whole Blood:

The cellular elements in whole blood were subsequently tested for their capacity to act as host cells for the replication of BHV-1 as follows:

Twenty-three and four tenth mls of bovine blood and the same volume

of fresh bovine plasma each was mixed with 2.6 ml of virus culture containing $10^{6.78}$ $TCID_{50}$ per ml. Three such preparations were made for the blood and also for the plasma control. The materials were placed in screw cap centrifuge tubes (20x125) and incubated at 4 C for 24 hours with occasional agitation at 3 hour intervals.

After thoroughly mixing the contents of the tubes two ml samples were withdrawn from each at the 24th hour. all samples were then centrifuged. The supernatant tractions contained unadsorbed virus. Such fractions were recovered and stored at -80 C . The tube contents were then remixed . All the tubes were immediately transfered to a waterbath held at 37 C and incubated for an additional 36 hours with occasional agitation.

Two ml samples were withdrawn at 3-hour intervals by gently centrifuging (150xg for 15 minutes) all the tubes and recovering 2.0 ml from the supernatants of each tube. The sediments were resuspended by flicking the bottom of each tube with a finger. The samples were stored at -80 C until sampling was completed, following which the virus content of each sample was determined by assay in BEL cell cultures in MT plate wells.

(ii) Red Blood Cells:

These cells were not tested for the capacity to serve as host cells for the virus.

(iii) Leukocytes:

The mononuclear cells were fractionated into glass adherent cells (possible progenitors of macrophages) and nonadherent cells. The latter cell population was taken as the equivalent of the lymphocytic component of whole blood, and separately tested for the capacity to support viral replication. Polymorphonuclear cells were examined in thin section microscopy only.

(a) Glass adherent mononuclear cells (AD-MNC) and bovine embryonic cells:

Identical cultures of adherent mononuclear cells (AD-MNC) and bovine embryonic lung cells (BEL) were used in this study in order to compare the virus growth supporting capacity of each cell type. This derives directly from the fact that both cell types are found in the lower respiratory passages and would be expected to encounter the virus during a part of the disease pathogenesis.

Briefly, five culture flasks (24 cm^2) were seeded with 9.5×10^6 AD-MNC per flask and 8.5×10^5 BEL cells per flask. The cells had been suspended in Eagles MEM, containing 20% FCS and antibiotics for AD-MNC, and 5% FCS for the BEL cells. The cells were allowed to attach to the glass by incubating the flasks at 37 C for 8 hours in an air incubator containing 5% carbon dioxide (CO_2) by volume. At this time all flasks were examined over an inverted microscope.*k All the cells in the BEL flasks had attached, but some cells in the AD-MNC flask had not. These cells were recovered by centrifuging the overlying fluid in each flask, and counting the cells in the resuspended sediments. Approximately 10% of the cells, on the average, failed to attach; thus each flask contained about $8.5 \times 10^{5.0}$ cells of AD-MNC, a number equivalent to that of the BEL cell cultures.

The fluid overlying the BEL cultures and that remaining on the AD-MNC cultures was decanted and the cell monolayers rinsed twice. The AD-MNC and the BEL cultures in each of three flasks per cell type were subsequently infected with BHV-1 at input multiplicities of 50:1 and 10:1, respectively. The monolayers in the remaining two flasks per cell type were overlaid with an equivalent volume of Eagle's MEM. They were used as cell controls.

---

*k American Optical Corporation, Buffalo, N. Y. 14215, Model 1810, Serial #; 139.

All flasks were held at 4 C for four hours after which the inoculums were decanted and the cells rinsed twice. The last washings from each cell type were pooled and a 10 ml volume sample was transferred to a flask correspondingly labeled with the cell type. All cells were subsequently overlaid with 10 ml of maintenance medium consisting of Eagle's MEM, fortified with 2% inactivated FCS (56 C for 30 mins.) and antibiotics. Eight-tenth ml samples were withdrawn immediately from each flask including those that contained infected control. All flasks were transferred to the incubator for the remainder of the determinations. Samples were withdrawn at 3 hour intervals through 36 hours and held at -80 C until all were collected.

Samples were tested for viral content by assaying for virus as described above.

(b) Non-adherent Mononuclear Cells (NA-MNC):

A suspension of NA-NMC containing 4.6×10^6 cells per ml of growth medium*1 was distributed in 5 ml amounts into each of three tubes. The cells were incubated at 37 C for 18 hours. A 1.0 ml dose of BHV-1 culture containing $10^{8.8}$ $TCID_{50}$ was added to each tube to provide an input multiplicity per cell of about 50. The final volume in each tube was 6.0 ml.

All tubes were incubated at 4 C for 6 hours. At this time the cells were sedimented by centrifugation at 150 x g for 15 minutes. One ml aliquots from the supernatants of each tube were recovered and stored for assay of residual virus.

---

*1 Eagle's MEM, fortified with 50% (V/V) of FCS, 100 I.U. penicillin; 100 mg streptomycin, and 75 mg gentimycin per ml, and 0.25 ml diluted phytohemagglutinin (PHA) Difco PHA-M (Gland Island Biological Company, 519 Aldo Avenue, Santa Clara, California 95050).

The cell sediments were rinsed twice. In each washing, fractions of the supernatant from all the tubes were pooled into one sample.

Finally, cells in each tube were resuspended in 14 ml of the same medium as used above, supplemented with 15% FCS and antibiotics. The cells were again sedimented and 4.0 ml samples from each tube withdrawn and pooled into a fourth tube for use as an inactivation control. Seven-tenth ml fractions were recovered from the supernatants in each tube at time zero. All tubes were then transferred to a waterbath maintained at 37 C. Similar samples were obtained at 12 hour intervals through a total of 144 hours. The 132nd hour sample was lost.

Titrations for viral content were done as for previous determinations.

(c) Polymorphonuclear Cells:

These cells were examined by electron microscopy only.

E. MORPHOGENESIS OF BOVINE HERPESVIRUS-I

The next series of experiments was performed to determine whether the morphogenesis of the virus in the blood cells is the same as that which occurs in indicator BEL cells routinely used for propagating the virus for diagnostic and other purpose and also to supplement the growth curve studies.

(i) Adherent Mononuclear Cells (AD-MNC) and Bovine Embryonic Lung (BEL) Cells:

Eight identical cultures of 2nd to 7th passage secondary cultures of BEL and freshly prepared glass-adherent mononuclear cells were grown in 16-oz. glass prescription bottles. The spent medium in corresponding bottles was decanted and cell cultures were rinsed twice with DPB. One and one half ml of virus culture containing $10^{6.78}$ $TCID_{50}$ per ml were added to each bottle giving an input multiplicity of 100:1. The bottles were incubated at 4 C for 4 hours to allow adsorption to occur. Thereafter

the virus inoculum in each bottle was decanted and the cell monolayers rinsed twice with DPB. The cells were then overlaid with 15 ml of maintenance medium consisting of Eagle's MEM supplemented with antibiotics and 5% and 20% FCS, respectively, for BEL and glass-adherent mononuclear cells. The bottles were transferred to a 37 C incubator. One bottle from each cell type was obtained at 0, 4, 6, 8, 10, 14, 18, 20 and 36 hours, and processed for electron microscopy (EM) as follows:

The overlying fluid was decanted and the cell monolayer rinsed twice in DPB. Five ml of 2% glutaraldehyde in Millonig's buffer was added to each bottle and the monolayers were held for 3 hours at 4 C for fixation to occur (cells could have been kept as long as possible in glutaraldehyde without ill-effects). Glutaraldehyde was decanted and the monolayers rinsed twice. The cells were pelleted and fixed in 1% osmium tetroxide (OS O_4) for 1 hour. Subsequently they were rinsed three times in Ringer's solution, 3 minutes per rinse, and afterwards stained in 0.5% uranyl acetate for 90 minutes. The cells were then rapidly dehydrated through 10-minute changes of increasing strengths (50%, 70%, 95%) of ethyl alcohol, followed by three 20 minute rises in 100% ethanol and two 15 minute rinses in propylene oxide.

The dehydrated specimens were infiltrated in 2:1, 1:1 and 1:2 series of a mixture of propylene oxide: Luft's Epon 812 (3:2, A:B*m) plus accelerator DMP-30*n 1% (55), 60 minutes each. The cells were then embedded

*m Resin Mixture

	Epon 812	DDSA	NMA	DMP-30
A	66 ml	100 ml	--	1% added
B	100 ml	---	84 ml	just before use

*n Abbreviations are:

DDSA: Dodecyl succinic anhydride
NMA: Nadic methyl anhydride
DMP-30: 2, 4, 6-tri (dimethyl amino methyl)-phenol

in 100% Luft's Epon 812 plus 1% DMP-30 accelerator and polymerized at 50 C for 48 to 72 hours. Thin sections were cut with Porter-Blum (Model MT-2), or Richert (Model O MU-2) ultramicrotomes and stained for three minutes in saturated uranyl acetate in 50% ethanol; washed in Ringer's solution and post-stained with Reynolds' lead citrate, diluted 1:1 with water for 3 minutes (83). The sections were examined and photographed in a Phillip's EM-200 electron microscope.

(ii) Non-adherent mononuclear cells:

Morphogenesis was followed in non-adherent cells recovered from fresh bovine blood and from blood that had been inoculated in vitro with the virus. Cells from fresh blood were prepared as outlined in the general cell separation method and were subsequently infected at an input multiplicity of 50:1. The cells were then incubated for 4 hours at 4 C and afterwards transferred to a water bath maintained at 37 C. Samples from infected cell preparations containing $8 \times 10^{7.0}$ cells total were obtained, washed twice in DPB and fixed in an excess of 2% glutaraldehyde by resuspending the cell pellets in the fixative. The rest of the procedure from this point onward is identical to that described for BEL and adherent mononuclear cells. When infected blood was used as the source of cells, the latter were fractionated on the Ficoll-Hypaque gradient as for the general cell separation procedure. Cell preparations were then serially adsorbed on glass and the non-adherent fractions were collected and processed for microscopy.

(iii) Polymorphonuclear Cells:

A similar approach as for the nonadherent mononuclear cells was followed for PMN cells, i.e. morphogenetic studies were done with isolated PMN cells as well as those recovered from infected blood. However, a third method was used in order to obtain infected PMN cells. Attempts were made to simulate conditions in an infected nasal passage into which PMN cells

might migrate in the course of a disease condition.

Briefly, monolayer cultures of BEL cells at the 2nd to 7th passage level were infected at an input multiplicity of 20:1. The cultures were incubated for 90 minutes at 4 C and subsequently transferred to a 37 C incubator after decanting the inoculum, rinsing the monolayer cells twice and adding fresh maintenance medium. The infected cells were then overlaid with a heavy suspension of PMN cells (8.6×10^7 cells per ml) and allowed to incubate further. The monolayers were closely examined every 90 minutes for cytopathologic changes (CPE). The PMN cells were recovered before full CPE occurred, or at the three time intervals of 9, 12 and 15 hours, whichever occurred first.

Cell preparations were then processed for microscopy as previously described.

III. RESULTS

A. CELL SEPARATION AND MAINTENANCE IN CULTURE:

The second layer in the separation tubes contained MN cells which were invariably more than 98% pure (500 cells examined); PMN cells were very few in number (1-2%). If RBC contamination occurred, it was usually less than 15 RBC per 100 leukocytes examined; the RBC were removed by lysis with deionized distilled water. Preparations of PMN cells were more than 95% pure; the usual contaminants were the MN cells, since the RBC were routinely removed during the preparation of the PMN cells. The monocytic cells adhered to glass very rapidly; the attachment regularly occurred within 1-3 hours' incubation at 37 C. The adherent cells were maintained in culture for periods varying from 5 - 28 days. Following attachment to glass, cells exhibited a spherical conformation. Their nuclei were barely discernible. Within 24 hours, the cytoplasm started separating into 2-3 slender processes which terminated in clubbed ends, or it spread around the nucleus in a single uniform mass. Thus, while some cells resembled fibroblasts in shape, ovoid and stellate figures were not uncommon. Adherent cells were ready to be infected with the virus within 5 days or more. When cells were kept for too long in culture, they shed protoplasmic processes and their nuclei became granular. The dead and dying cells soon detached from the glass.

In the non-adherent cells, PHA was required for continued viability in cultures maintained for more than 4 days. Hence, any time nonadherent cells were not used immediately following isolation, they were kept frozen (at -80 C) suspended in a mixture consisting of 15% DMSO*o and 15% FCS

---

*o Dimethyl sulfoxide, Mallinkrodt Chemical Works, St. Louis, Miss. 63147.

(V/V in Eagles MEM) at 5 x 10^7 cells per ml concentration. Frozen cells were rapidly thawed and washed once before use. More than 85% of the cells were viable after 3 weeks' storage.

B. ADSORPTION: WHOLE BLOOD AND SEPARATED CELLS:

In whole blood and separated cell components more virus was adsorbed from the smaller of the two viral inoculums. Figure (3) shows the percent adsorption in the various test preparations. It is apparent that no adsorption occurred in whole blood after one hour of incubation. However, adsorption could be detected in subsequent time intervals reaching a maximum level at 18 hours. Afterwards there was a gradual decline in viral adsorption. The percent adsorption in the leukocytic fraction was about 30% of maximum after 1 hour of incubation with a subsequent peak at 24 hours. The unfractionated leukocyte preparations possessed the highest capacity to adsorb the virus for all the cell preparations tested. The MN cells showed an appreciable adsorption activity at 1 and 6 hours, followed by a slight drop and then a subsequent rise. The rates in the RBC component were one half to one third as much or smaller as compared to the corresponding values for the MN cell fractions. The RBC's adsorbed no virus within the first hour of incubation. However, some adsorption had occurred within 6 hours after which there was an apparent elution at 12 hours followed by a reduced rate of viral adsorption. It seemed as though some virus also eluted from the MN cells at the 12th hour.

C. REPLICATION:

(i) Growth in whole blood:

Data from the adsorption experiments showed that BHV-1 was adsorbed by cellular elements in whole blood and that the various cellular components adsorbed unequal varying quantities of the virus. This also indicated that the cells adsorbed the virus at correspondingly different rates. Subsequent

experiments were done to determine whether the virus would replicate in whole blood or in the various isolated cellular components. Figure (4) shows that the virus eclipses within 3 hours, followed shortly by an exponential growth phase. The virus titer reached a peak of 1×10^5 $TCID_{50}$ per tube at approximately 15 hours, with a subsequent decline. Freshly prepared bovine plasma, inoculated with an identical viral dose as that inoculated into the blood had been set up as the non-specific viral inactivation. Figure (4) shows that the virus titer in the control plasma tubes gradually decreased over time. Within the first 9 hours, while the titer in whole blood was steadily increasing, that in the plasma control was at a higher gradually decreasing value for each corresponding time interval. This indicates that viral inactivation was occurring even during the period of exponential viral replication.

Results from this experiment are adequate evidence that BHV-1 readily multiplied in cells found within whole blood. The next question was, "In which cell types would viral replication occur within the blood?" Data from the adsorption studies had shown that the unfractionated leukocytic components adsorbed the most virus of the cell types tested. It seemed worthwhile to separate these cells into the various components and test each for its ability to support replication of BHV-1. Therefore, PMN and MN cell preparations were obtained. The latter were further fractionated into glass-adherent (macrophage equivalents) and non-adherent (lymphocytic equivalents) cells prior to inoculation with the virus.

(ii) Separated cells:

(a) Growth in BEL and Adherent Mononuclear cells:

Figure (5) shows that the maximum viral titer attained at 12 hours not only indicated the end of an exponential growth phase in the adherent MN cells, but also the start of a similar decline in viral titer. After 24

hours there was little detectable virus in these preparations. The reduction in viral titer in samples obtained from the later incubation periods could be attributed to heat inactivation and to the reduction in the number of viable cells that were actively producing infectious virus. However, it could also be postulated that the bell-shaped curve is peculiar to a mononuclear cell system in comparison to other cell systems. To test the validity of this proposal, an identical dose of the virus was inoculated into several 4 hour-old cultures of BEL cells containing the same cell concentration per flask as for the adherent MN cells used in the preceding experiments. A similar sampling procedure was followed. The data (fig. 5) indicated that for the fist 6 hours there was very little infectious virus released by the BEL cells. However, there was a rapid release of infectious virus shortly thereafter, starting at the 9th hour and continuing through the peak period at the 18th hour.

In general, there were two notable differences between these cell types: 1. that viral replication in the adherent cells was rapid, with a peak being 2 log units below that of BEL cells. The virus eclipse period was rather indistinct in the adherent cells. 2. Conversely that there was a 6-hour eclipse period in the BEL cells followed by maturation and release, phenomena which were much clearer in the BEL than in the adherent MN cells.

(b) Nonadherent MN cells:

The data (fig. 6) shows that these cells can support viral replication following stimulation with PHA. The peak titer was observed at 36 hours followed by a gradual decline. If we recall that the peak titer in the BEL cells was at 18 hours, that in the non-adherent cells might seem delayed. The apparent delay could indicate that although the viral inoculum was sufficient to infect all the cells, yet, these cells were asynchronously infected. It might also mean that the growth curve represented multiple

replication cycles. In any case, the nonadherent MN cells yielded the least viral titers of all cell types tested. However, since the BEL cells were grown as monolayers while the lymphocytic cells were maintained in stationary suspension cell cultures, it is advisable to be cautious when making these comparisons.

In another experiment, infected nonadherent MN cells were maintained in MT plates and examined periodically over an inverted microscope. There were no significant morphologic differences between infected and non-infected cells during the first 5 hours. However, at about 6 hours post infection, the infected cells began to lose the original characteristic lusture of healthy cells. At first the cell membranes were smooth, and each cell was rounded; at the 6th hour, the cell membranes were irregular, distorting the cell into bizarre shapes. Within 9 hours of incubation many of the cells had aggregated into clumps of 3 to 4 or more cells, although some remained single. Cell populations examined 48 hours after infection were found to contain some ballooning and degenerating cells while extensive fusion of cells had occurred. Electron micrographs of infected cells taken at the identical time intervals as above showed that from 14 to 20 hours, the nucleoplasm of some of the cells had undergone marked reduction in electron density, with concomitant condensation and margination of nuclear chromatin. The nuclei of other cells had undergone complete disintegration. Surprisingly, one could see a large number of uninfected cells together with infected ones. The former exhibited completely normal structural detail (fig. 22). It is clear from these data that not all cells became infected, and that probably most of the infected cells did not release much virus.

(c) Polymorphonuclear cells:

When these cells were tested for the capacity to support viral

replication , no increase in viral titer was noted. However, electron micrographs of thin sections of PMN cell preparations were examined and the results are reported below.

D. MORPHOGENESIS:

(i) BEL cells:

Since the object of the study was to examine more intensively the leukocytic cell-virus interactions, the morphogenetic studies were done in greater detail in the leukocytic than in the BEL cells. The morphology of viral particles observed during the examination of sections of samples from the leukocytic cell components was compared to that observed in BEL cells. Viral nucleocapsids were first seen in the nuclei of the latter cells from the 5th hour samples; subsequent samples obtained at 8 hours and at later intervals revealed fully mature enveloped particles in addition to intranuclearly located viral nucleocapsids (figs. 7,8).

(ii) Adherent MN cells:

Nucleocapsids were not observed during the first 5 hours in these cells. However, cells infected and examined after 4 hours appeared to contain increased amounts of electronlucent central nucleoplasm , but no viral nucleocapsids (fig. 9). The cytoplasm of these cells contained a well developed rough endoplasmic reticulum and Golgi apparatus, indications of protein synthesis at the time of sampling. Poorly resolved, intracytoplasmic membrane-bound inclusions were also present in these cells, but they contained no viral particles. Nucleocapsids were seen in the nucleus for the first time at 6 hours after infection. Similar nucleocapsids were also seen 8 hours after infection (fig. 10, 11) while mature viral particles were observed in the 8th hour samples and consistently thereafter (fig. 12, 13). In a cell from samples harvested 14 hours after infection, several

nucleocapsids were seen in the nucleus as well as some viral particles probably budding into cytoplasmic vacuoles (fig. 14).

If the MN cells were maintained for 21 days before infection, unstained preparations examined by light microscopy showed that in the late stages of incubation, the nuclei became granular, pyknotic and eventually detached from the glass surface, leaving behind the firmly attached cytoplasm. Figure 15 shows an electron micrograph of a cell from cultures exhibiting marked detachment of cell nuclei. They had been infected with BHV-1 9 hours previously. Such cells contained abundant multivesicular cytoplasm, and the cytoplasmic vacuoles contained cellular debris from the overlying fluid. Numerous clubbing acanthopodes were seen on the cell membranes of most of these cells.

(iii) Nonadherent MN cells:

Figure 17 shows cells incubated for 14 hours after inoculation with BHV-1. They contained viral nucleocapsids. No viral particles were seen in the cytoplasm or in the process of being released. Cells from several other samples obtained from similar culture preparations at 14 hours after infection did not contain viral particles either in the nucleus or in the cytoplasm (fig. 18). A similar erratic distribution of viral particles was observed in the samples obtained at 18 and 20 hours postinfection (figs. 19 - 24). Samples from the 36th hour and later incubation periods consistently revealed the presence of viral particles (figs. 25 & 26). This erratic distribution indicated that the nonadherent MN cells were not uniformly susceptible to the virus. This would also be consistent with a multiphasic growth curve.

(iv) Polymorphonuclear cells:

When a suspension of PMN cells and virus was mixed, incubated at 37 C and samples withdrawn at various time intervals and examined for virus, none

was observed in the samples prior to the 48th hour of incubation. All such samples contained cells with electrondense peripheral chromatin, while the centrally located chromatin was more electronlucent. The nuclear membrane was very clearly demarcated from the cell cytoplasm. The latter contained lysosomal granules of varying sizes. Several nuclear lobes could be seen in sections of many cells. This was consistent with the segmented nuclei observed in PMN cells of peripheral blood in light micrscopy (fig. 27).

The cells from the 48th hour samole (fig. 28) contained many cytoplasmic multivesicular vacuoles and an abundance of monovesicular ones of varying sizes. Both types of vacuoles contained materials of various sizes and shapes, probably at different stages of degradation. No lysosomes could be clearly identified in these cells. However, it was assumed that the lysosomes had fused with the phagocytic vacuoles to form phagolysosomes, hence explaining the presence of the multivesicular vacuoles, and the absence of the lysosomes. Whether the lysosomes disappeared because of fusion, as suggested above, or due to the prolonged in vitro cultivation of PMN cells is debatable. The nuclei contained viral particles which, at higher magnification (fig. 29), seemed to be clearly herpesviral nucleocapsids. There was no indication of viral maturation or release by these cells . The latter appeared to contain more areas of electronlucent nuclear chromatin than those cells in which viral particles were not seen.

The PMN cells that were co-cultivated with infected BEL cells did not contain viral particles in their nuclei at any stage of incubation. However, the phagocytic vacuoles contained cellular debris, probably from the BEL cells, and some viral particles (figs. 30 & 31). It seems that these viral particles were noinfectious to the PMN cells. Most of the viral particles were probably degraded by lysosomal hydrolases together with the rest of the phagocytosed cellular debris.

In another PMN cell preparation, the poorly fixed lysosomes contained "onion peel", whorl-like structures, whereas well fixed lysosomes exhibited a fibrillar internal structure (fig. 32).

IV. DISCUSSION

A number of methods have been used to separate the cellular elements of blood. The techniques include the use of simple centrifugation to obtain the buffy coat cells, followed by lysis of the contaminating red blood cells (12, 22, 76), attachment of the buffy coat cells to Roux bottles (29), density gradient centrifugation (48, 99), zonal centrifugation (9), a combination of red cell aggregation and density gradient sedimentation (10, 80); or by the use of glass or polystylene bead columns (81, 98). The method of Böyum (10) was modified for use in this study. Good results were obtained utilising this method as shown by the high viability and purity of cell preparations recovered in this study.

In order to understand the nature of the interaction of a virus with host cells, it is imperative to subdivide the viral replication cycle into phases that could be studied easily. By introducing factors that interrupt the cycle at defined or definable points, much has been learned regarding the influence of the infecting virus on the host cell metabolism, and the sequence of events that lead to the production of progeny virus. Five merging phases of the viral replicative cycle have been identified. These are: 1. Adsorption 2. Penetration 3. Uncoating 4. Biosynthesis 5. Maturation and release. The events which occur during each phase differ widely, depending on the type of virus and the host cell. A defect in any step can result in an abortive or defective replicative cycle. This might explain why host cells are either permissive or non-permissive to infection with different viruses.

In the present study, only the adsorption, maturation and release phases were examined. It was hoped that the data from such a study would provide evidence to support or refute the validity of the hypotheses advanced in the introduction. The finding that adsorption by cells in whole blood was less than in the fractionated cell component was at first puzzling. However, if we recall that identical volumes of whole blood and isolated cell components were used, this would seem to be a consistent finding. This is because volume for volume, whole blood would contain fewer cells than an equivalent volume of the concentrated preparations of separated cell components. Intuitively, one would expect more cells to adsorb more virus than if a few cells were used. This intuition is upheld by the data.

To some extent, the rate of adsorption of herpes simplex and pseudorabies viruses is thought to be dependent on the relative cell concentrations. For example, Farham (28) found that when the same amount of virus was added to cultures in volumes of 1.0 ml, 0.5 ml or 0.1 ml, the relative number of plaques obtained was 1, 1.5, and 1.7. One can say that the adsorption of BHV-1 like that of the other two herpesviruses, is also dependent on the cell concentration. When Stevens and Groman (92) studied the adsorption of BHV-1 to monolayer cultures of the Madin-Darby bovine kidney (MDBK) cells, and assayed the adsorbed virus by the plaque technique, they observed greater adsorption of virus from the smaller of the two inoculums used. However, no work has been reported on the adsorption of BHV-1 to leukocytes; neither has much been done on the adsorption phase in this general phenomenon of cell-virus interactions between leukocytic cells and viruses, except that done with influenza virus (2, 3, 34). Nevertheless, the growth of many viruses in leukocytic cells is widely reported (21, 22, 23, 24, 38, 74, 79, 91).

In the present study, small quantities of BHV-1 were adsorbed by the red cells. The adsorption per se was difficult to explain. Since herpesviruses replicate in the nuclei of infected cells, no viral replication would be expected to occur in anucleate red cells. Therefore, the adsorption would be nonspecific. Poliovirus (69), influenzavirus (2) and Newcastle Disease virus (85) have been shown to be unproductively attached to cells in culture. Part of the adsorbed poliovirus was shown to be infectious upon subsequent retesting (69). Since viral elution was observed in the red cell series in the present study as judged by the increased infectious residual virus in the later hours of incubation, it was alluded that the eluted BHV-1 was infectious.

The greater viral adsorption in the unfractionated leukocytic components than in the separated mononuclear cells is a consistent finding since the former fraction also contained PMN's. The latter cells would be expected to adsorb some virus as well. Although direct evidence for viral adsorption to PMN's was unavailable, electron micrographs indicated that these cells phagocytized viral particles (fig. 31). However, it is evident from the adsorption and also from the viral replication studies that both types of MN cells adsorbed considerable quantities of the virus.

Valentine and Allison (3) have shown that the rate of adsorption of labelled influenza virus decreased after about 60% of the virus had adsorbed. They attributed the decline to the clumping of cells in the later hours of incubation. They also demonstrated that it was not due to the presence of a proportion of viral particles less easily adsorbed to the cell suspension by mixing supernatants from a late incubation sample with a fresh cell suspension. They found the rate of adsorption to be similar to that initially observed. The decrease in viral adsorption over time in the present study was attributed to cell clumping. It is well established

that 100% adsorption of inoculum virus is not essential to ensure viral infection of a cell suspension or a monolayer. It is also a common practice in virological work to rinse off excess inoculum virus seeded to monolayer cultures, or to wash infected cell suspensions during growth curve studies. This is a clear indication of a general presumption that only a small portion of inoculum virus is essential for the infection of any desired indicator cells. In the present study, viral replication was observed in the mononuclear cell types although a hundred percent viral adsorption did not occur.

As indicator cells for viral replication, leukocytic cells would be either permissive or nonpermissive to the viral infection. However, in addition , they would also have the capacity to actively destroy the virus. It was hoped to show by these studies that leukocytes adsorb large amounts of virus. It would have been of interest to compare adsorption by the leukocytes and by an equal quantity of trypsinised BEL cells.

While it is difficult to demonstrate viral replication in the blood stream, many viruses have been shown to replicate in vitro in blood cell cultures of birds (11, 21), lower animals (21, 22, 30, 57), and man (8, 23, 24, 26, 30, 38, 74, 102). The presence of viruses in blood cells has been demonstrated in clinical materials using fluorescent antibody (FA) techniques (15, 97), by direct isolation from leukocytes of various species (27, 37, 39, 50, 66, 67) or by electron-microscopic (EM) examination (11, 26, 79).

When whole bovine blood was inoculated with BHV-1, viral replication occurred. It occurred also in some of the cellular fractions of whole blood. Since stimulation with PHA was necessary before lymphocytes could support viral replication, these cells were considered unlikely to act as host cells for viral replication in vivo. However, lymphocytes could

readily adsorb the virus (table 1). Since growth curve studies showed that PMN cells did not allow viral replication and that only rarely were nucleocapsids demonstrated in these cells, it was concluded that they are unlikely to contribute significantly to viral titers in whole blood. It is also believed that the PMN cells would internalise and inactivate any virus adsorbed to their surfaces. Evidence from the growth curve and EM studies strongly indicated that the monocytes acted as host cells for viral replication without stimulation with PHA. Since there is no known *in vivo* equivalent of PHA stimulation, except that due to specific antigens and neoplasia, it was concluded that only monocytes could support viral replication *in vivo*.

From the adsorption and the replication studies, it is concluded that *in vivo*, the virus is transported replicating in monocytes and adsorbed to the lymphocytic cell surfaces. This is based on the fact that although both cell types adsorbed the virus, only the monocytes could replicate the virus without the aid of blastogenesis, and that while lymphocytes with or without PHA adsorbed the virus, only the former supported viral replication. It is conceivable that the non stimulated lymphocytes retained the virus on their cell surfaces.

It is not possible to isolate virus from whole bovine blood always because only small quantities are released. This leads to viral titers that are below the detection threshold for the isolation techniques currently in use. Also, lymphocytes adsorb the virus further reducing the virus titer. Furthermore, only about 5% of the leukocytic cell components are monocytes, again diminishing the possibilities of viral detection. However, if buffy coat cells are cultured, the viral concentration would be adequate to allow detection.

Since the manner of viral replication in the blood cells differed significantly from that observed in BEL cells, I will try to explain the possible sources of these differences. As shown by the growth curve studies, virus yield was lower in adherent MN cells than in identical cultures of BEL cells. The fewer number of nucleocapsids in the nuclei of adherent cells as compared to those of BEL cells could explain this observation. A reduction in the envelopment in the blood cells, particularly in the nonadherent types could have lead to a reduction of viral maturation and release. In this regard, the apparent restriction on viral replication would not be due to influences in the early phases of the infection cycle, i.e. adsorption, penetration or uncoating, but by changes or factors that could alter the subsequent biosynthesis, maturation or release. Thus, while the regulation of cellular metabolic pathways in BEL cells might be completely taken over by the infecting virus, the blood cells could retain control over their metabolic pathways and act in a manner that would restrict viral production. The cells could do this by adversely influencing the transcription of viral genome or the translation of viral coded information, or by restricting viral assembly and release. Also we may recall that these cells are equipped with degrading enzymes which may destroy the virus. In the final analysis this merely means that less virus is produced than in the case of other cells.

Another way in which viral restriction could occur in blood cells is via the release from the activated macrophages or lymphocytes, of a variety of factors that could render the susceptible cells refractory to infection, or suppress viral replication in the infected cell. This mechanism is applicable in the present study. It might be that in both the adherent and nonadherent cell fractions the cells that contacted the virus initially

elaborated an inhibitory substance, like interferon which, on reaching the other cells, reduced viral yield in the latter cells.

Suppression of BHV-1 growth in indicator cells by murine macrophages has been documented (84), but no work on such suppression by bovine macrophages or lymphocytes has so far been reported. Stevens and Cook (91) concluded that the reduction in viral growth of herpes simplex in murine macrophages was probably at the deoxyribonucleic acid (DNA) synthesis level. They suggested that defective progeny viral DNA or lesser amounts of normal viral DNA were synthesized in macrophages that showed restriction of herpesviral replication. Johnson (49) showed that infected murine macrophages from young mice released virus to adjacent cells cocultivated with the macrophages, whereas there was restriction of viral release in macrophages from adult mice. This indicated that there was interference with viral release in the latter cells. Data from the present study indicated that substantial virus was released from cultured bovine leukocytes other than PMN cells. Thus, restriction of viral release would not explain the reduction in viral titer observed in these leukocytic cultures.

The morphogenetic studies in both the adherent and the nonadherent mononuclear cells confirmed the results of the growth curve studies by showing that BHV-1 replicated in these cells. The absence of mature viral particles within 5 hours' incubation is consistent with the presence of an eclipse phase in the corresponding time interval in the growth curve. Mature virus particles were observed in the 8-hour samples of adherent cell preparations, a period which corresponds to the beginning stages of the exponential phase of the growth curve, again, a consistent finding.

The nonadherent cells were the most unique in their capacity to act as host cells for BHV-1, in that non-PHA stimulated cells failed to support viral replication. The electron micrographs showed that some of the PHA

stimulated cells failed to support viral replication. Thus, the growth curve (fig. 6) might not necessarily be a one step growth cycle. Although no receptors have been identified for herpesviruses, it is conceivable to assume, based on the work done with other viruses that such structures exist (107). If this is true, the effect of PHA on the lymphocytes could be, in part, to unmask such receptors or cause their synthesis, in addition to the well known effect of blastogenic transformation. Non stimulated cells would lack such receptors and hence escape viral infection. Transformed lymphocytes show increased DNA synthesis, while the DNA of untransformed cells is inactive. It would seem logical to conclude that viral replication occurred in the one cell type and not in the other because of the respective active and inactive DNA metabolism.

Summary and Conclusions

The leukocytic fraction of whole blood was shown to adsorb the largest quantities of BHV-1, while the adsorption by the red cells was considered insignificant. Electron microscopic evidence showed indirect viral adsorption by the PMN's. Both types of mononuclear cells adsorbed the virus. However, viral replication accompanied by release of virus occurred only in the monocytic cells and PHA stimulated lymphocytes.

It was concluded that BHV-1 is transported in the blood replicating in monocytes and adsorbed to lymphocytic cell surfaces.

BIBLIOGRAPHY

1. Abinauti, F. R. and Plumer, G. J.; The Isolation of Infectious Bovine Rhinotracheitis virus from Cattle Affected with Conjunctivitis - Observations on the Experimental Infection. Am. J. Vet. Res. 22, (1961):13-17.

2. Ackermann, W. W., Ishida, N. and Maasab, H. F.; Growth Characteristics of Influenza Virus Concerning the Binding of Virus to Host Cells. J. Exp. Med. 102, (1955):545.

3. Allison, A. C. and Valentine, R. C.; Virus Particle Adsorption: II. Adsorption of Vaccinia and Fowl-plague Viruses to Cells in Suspension. Biochimica et Biophysica Acta, 40(1960):393-399.

4. Allison, A. C. and Valentine, R. C.; Virus Particle Adsorption: III. Adsorption of Viruses by Cell Monolayers and Effects of Some Variables on Adsorption. Biochimica et Biophysica Acta, 40(1960):400-410.

5. Bailey, N. T. J.; Mathematical Approach to Biology and Medicine. Wiley, N. Y.; 1967.

6. Baker, James A., McEntee, Kenneth, and Gillespie, J. H.; Effects of Infectious Bovine Rhinotracheitis-Infectious Pustular Vulvovaginitis (IBR-IPV) Virus on Newborn Calves. Cornell Vet., 50(1960):156-170.

7. Barenfus, Morris, Delli Quadri, C. A., McIntyre, R. W. and Schroeder, R. J.; Isolation of Infectious Bovine Rhinotracheitis Virus from Calves with Meningoencephalitis. J.A.V.M.A., 143(1963): 725-728.

8. Berg, R. B. and Rosenthal, M. S.; Propagation of Measles Virus in Suspensions of Human and Monkey Leukocytes. P.S.E.B.M., 106 (1961):581-585.

9. Boone, C. W., Harell, G. S. and Bond, H. E.; The Resolution of Mixtures of Viable Mammalian Cells into Homogeneous Fractions by Zonal Centrifugation. J. Cell Biol., 36(1968):369-378.

10. Böyum, A.; Isolation of Mononuclear Cells and Granulocytes from Human Blood. Scand. J. Clin. Lab. Invest. 21 (suppl. 97):77-89.

11. Campbell, J. G. and Woode, G. N.; Demonstration of a Herpes-type virus in Short-term Cultured Blood Lymphocytes Associated with Marek's Disease. J. Med. Microb., vol. 3, (1970):463-473.

12. Carlson, G. P. and Kaneko, J. J.; Isolation of Leukocytes from Bovine Peripheral Blood. P.S.E.B.M., vol. 142, (1973):853-856.

13. Cawley, J. C. and Hayhoe, F. G. J.; Ultrastructure of Haemic Cells. Cytological Atlas of Normal and Leukaemic Blood and Bone Marrow. W. B. Saunders, Co., Ltd., Philadelphia, 1973.

14. Carrel, A. and Ebeling, A. B.; Pure Cultures of Large Mononuclear Leukocytes. J. Exp. Med., vol. 36, (1922):305-377.

15. Cello, R. M., Moulton, J. E. and McFarland, S.; The Occurence of Inclusion Bodies in the Circulating Neutrophils of Dogs with Canine Distemper. Cornell Vet. 49, (1959):127-146.

16. Chennekatu, P. Peter, Gratzek, John B. and Ramsey, Frank K.; Isolation and Characterisation of a Strain of Infectious Bovine Rhinotracheitis Virus Associated with Enteritis in Cattle: Pathogenesis Studies by Fluorescent Antibody Tracing. Amer. J.Vet. Res. vol. 27, (1966) : 1583-1590 .

17. Christian, Robert T. and Ludovici, Peter P.; Cell-to-cell Transmission of Herpes Simplex Virus in Primary Human Amnion Cells. P.S.E.B.M., vol. 138(1971):1109-1115.

18. Dales, Samuel and Silverberg, Harriette; Viropexis of Herpes simplex virus by Hela Cells. Virol., 37, #3(1969):475-480.

19. Roizman, Bernard and Spear, Patricia; Herpes viruses, In "Ultrastructure of Animal Viruses and Bacteriophages":An Atlas. Edited by Albert J. Dalton and Francoise Haguenau. Acad. Press, New York, 1973, pages: 83-107.

20. Deas, D. W. and Johnston, W. S.; The Isolation and Transmission of Infectious Bovine Rhinotracheitis-Infectious Pustular Volvovaginitis. Vet. Rec., 92(1973):636-639.

21. Dunne, H. W., Luedke, A. J. and Hokanson, J. I.; The Growth of Animal Leukocytes and Their Use in the Cultivation of Animal Viruses. Amer. J. Vet. Res., vol. 19, (1958):706-711.

22. Dunne, H. W., Luedke, A. J., Reich, C. V. and Hokanson, J. F.; The In vitro Growth of Hog Cholera Virus in Cells of Peripheral Blood. Amer. J. Vet. Res., vol. 18, (1957):502-507.

23. Edelman, Robert and Wheelock, E. Frederick; Specific Role of each Human Leukocyte Type in Viral Infections. I. Monocyte as Host Cell for Vesicular Stomatitis Virus Replication In vitro. J. Virol,, vol. 1, (1967):1139-1149.

24. Edelman, Robert and Wheelock, E. Frederick; Specific Role of each Human Leukocyte Type in Viral Infections; II. Phytohemagglutinin-treated Lymphocytes as Host Cells for Vesicular Stomatitis Virus Replication In vitro. J. Virol., vol. 2, (1968):440-448.

25. Emmens, C. W.; Principles of Biological Assay. William Clowes and Sons Ltd., London and Beccles, 1948.

26. Epstein, M. A.; Achong, B. G., and Barr, Y. M.; Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. Lancet ii, (1964):702.

27. Falk, Lawrence A., Wolfe, Lauren G. and Deinhardt, Friedrich; Isolation of Herpesvirus Saimiri from Blood of Squirrel Monkeys (Saimiri Sciureus). J.N.C.I., vol. 48, #5, (1972):1499-1505.

28. Farnham, A. E.; The Formation of Microscopic Plaques by Herpes Simplex virus in Hela Cells. Virol. 6(1958):317-327.

29. Fitzgeorge, R. B., Solotorovsky, M. and Smith, H.; The Behavior of Brucella abortus Within Macrophages Separated from the Blood of Normal and Immune Cattle by Adherence to Glass. Brit. J. Exp. Path. 68(1967):522-528.

30. Florman, A. L. and Enders, J. F.; The Effect of Homologous Antiserum and Complement on the Multiplication of Vaccinia Virus in Roller Tube Cultures of Blood Mononuclear Cells. J. Imm. 43, (1942): 159-174.

31. Franklin, R. M.; The Growth of Fowl plague virus in Tissue Cultures of Chicken Macrophages and Giant Cells. Virol. 6, (1958):81-95.

32. French, E. L.; Relationship Between Infectious Bovine Rhinotracheitis (IBR) virus and a Virus Isolated from Calves with Encephalitis. Aust. Vet. Jour., vol. 38, (1962):555-556.

33. Gillespie, J. H., McEntee, K., Kendrick, J. W. and Wagner, W. C.; Comparison of Infectious Pustular Vulvovaginitis virus with Infectious Bovine Rhinotracheitis virus. Corn. Vet. 49(1959): 288-297.

34. Ginsberg, Harold S. and Blackmon, John R.; Reactions of Influenza Viruses with Guinea Pig Polymorphonuclear Leukocytes, I. Virus-cell interactions. Virol., 2(1956):618-636.

35. Gratzek, J. B., Chennekatu, P. B. and Ramsey, F. K.; Isolation and Characterisation of a Strain of Infectious Bovine Rhinotracheitis virus Associated with Enteritis in Cattle: Isolation, Serologic Characterisation and Induction of Experimental Disease. Amer. J. Vet. Res., vol. 27, (1966):1567-1572.

36. Gratzek, J. B., Jenkins, Robert A., Chennekatu, P. Peter and Ramsey, Frank K.; Isolation and Characterisation of a Strain of Infectious Bovine Rhinotracheitis virus Associated with Enteritis in Cattle: Comparative Development Study by Fluorescent Antibody Tracing and Electron Microscopy. Amer. J. Vet. Res., vol. 27, (1966):1573-1582.

37. Gresser, Ion and Chany, Charles; Isolation of Measles virus from the Washed Leukocytic Fraction of Blood. P.S.E.B.M., 113(1963): 695-698.

38. Gresser, I. and Chany, C.; Multiplication of Poliovirus Type-1 in Preparations of Human Leukocytes and its Inhibition by Interferon. J. Imm., vol. 92(1964):889-895.

39. Henle, G. and Henle, W.; Evidence for a Persistent Viral Infection in a Cell Line Derived from Burkitt's Lymphoma. J. Bact. 89(1965): 252-258.

40. Hirsch, M. S., Zisman, B. and Allison, A. C.; Macrophages and Age Dependent Resistance to Herpes simplex virus in Mice. J. Imm., vol. 104(1970):1160-1165.

41. Hitt, C. W.; Kinetics of Virus Inactivation. Bact. Rev., vol. 28 (1964):150-163.

42. Hochberg, Edna and Becker, Y.; Adsorption, Penetration and Uncoating of Herpes simplex virus. J. Gen. Virol. 2, (1968):231-241.

43. Holland, John J. and Mclaren, Leroy C.; The Mammalian Cell-Virus Inter-relationships: II. Adsorption, Reception and Eclipse of Polio virus by Hela Cells.

44. Holman, H. H.; Biological Research Methods; Practical Statistics for Non-mathematicians. Hafner Publishing Company, New York, 2nd Ed., 1969.

45. Hughes, J. P., Olander, Harvey J. and Wada, E. M.; Keratoconjunctivitis Associated with Infectious Bovine Rhinotracheitis. J.A.V.M.A., vol. 145, #1(1964):32-39.

46. Irwin, J. O. and Cheeserman, E. A.; On an Approximate Method of Determining the Median Effective Dose and its Error in the Case of a Quantal Response. J. Hyg. 39(1939):574.

47. Jasty, V. and Chang, P.; Envelopment of Infectious Bovine Rhinotracheitis viral Particles in Bovine Kidney Cell Cultures: An Electron Microscopic Study. Amer. J. Vet. Res., 32, (1971): 1945-1953.

48. Joel, D. D., Adamik, E. R., Chanana, A. D., Cronkite, E. P., Shiffer, L. M. and Sipe, C. R.; Separation of Lymphocytes from the Blood of Calves and Goats. Am. J. Vet. Res., vol. 30, (1969): 1099-1104.

49. Johnson, R. T.; The Pathogenesis of Herpesvirus Encephalitis. II. A Cellular Basis for the Development of Resistance with Age. J. Exp. Med. 120, (1964):359.

50. Kemey, L. and Pearson, James E.; Isolation of Herpesvirus from Equine Leukocytes: Comparison with Equine Rhinopneumonitis virus. Can. J. Comp. Med., Vol. 34, (1970):59-65.

51. Kendrick, J. W., McKercher, D. G. and Saito, J.; Preliminary Report of Studies on a Catarrhal Vaginitis of Cattle. J.A.V.M.A., vol. 128(1956):357-361.

52. Kendrick, J. W., Schneider, L. and Straub, O. C.; Placental Reaction to the Infectious Bovine Rhinotracheitis-Infectious Pustular Vulvovaginitis virus. Amer. J. Vet. Res. 32, (1971):1045-1051.

53. Kendrick, J. W. and Straub, O. C.; Infectious Bovine Rhinotracheitis-Infectious Pustular Vulvovaginitis virus Infection in Pregnant Cows. Am. J. Vet. Res., vol. 28, #126(1967):1269-1282.

54. Kennedy, P. C. and Richards, W. P. C.; The Pathology of Abortion Caused by The Virus of Infectious Bovine Rhinotracheitis. Path. Vet. 1, (1964):7-17.

55. Luft, J. H.; Improvements in Epoxy Resin Embedding Methods. J. Biophys. Biochem. Cytol., 9(1961):409-414.

56. Madin, S. H., York, C. J. and McKercher, D. G.; Isolation of the Infectious Bovine Rhinotracheitis virus. Science, 124(1956): 721-722.

57. Malquist, W. A. and Hay, D.; Hemadsorption and Cytopathic Effect Produced by African Swine Fever virus in Swine Bone Marrow and Buffy Coat Cultures. Am. J. Vet. Res. 21(1960):104-108.

58. Marchesi, V. T.; The Site of Leukocyte Emigration During Inflammation Quart. J. Exp. Physiol., 46(1961):115.

59. Marchesi, V. T. and Florey, H. W.; Electron Micrographic Observation on the Emigration of Leukocytes. Quart. J. Exp. Physiol., 45 (1960):343.

60. Marchesi, V. T. and Gowans, J. L.; The Migration of Lymphocytes Through the Endothelium of Venules in Lymph nodes: an Electron Microscope Study. Proc. Roy. Soc. Ser. B., 159(1964):283.

61. Mare, John and Van Rensburg, S. J.; The Isolation of Viruses Associated with Infertility in Cattle: A Preliminary Report. J. South African Vet. M. A., 32, (1961):201-210.

62. LaVia, Mariano F. and Hill, Rolla B., Jr., editors; Fate of Inflammatory Cells In "Principles of Pathobiology". Oxford University Press. New York, Toronto, (1971):115-118.

63. McKercher, D. G., Bibrack, Barbara and Richards, W. P.; Effects of the Infectious Bovine Rhinotracheitis virus on the Central Nervous System of Cattle. J.A.V.M.A., vol. 156, #10, (1970): 1460-1467.

64. McKercher, D. G., Moulton, J. E., Kendrick, J. W. and Saito, J.; Recent Developments on Upper Respiratory Diseases of Cattle. Proc. 59th Ann. Meeting U.S.L.S.A. (1954) (1955):151.

65. McKercher, D. G., Straub, O. C., Saito, J. K. and Wada, E. M.; Comparative Studies of the Aetiological Agents of Infectious Bovine Rhinotracheitis and Infectious Pustular Vulvovaginitis. Can. J. Comp. Path. and Med., vol. 23, #10(1959):320-328.

66. McKercher, D. G. and Wada, E. M.; The Virus of Infectious Bovine Rhinotracheitis as Cause of Abortion in Cattle. J.A.V.M.A., vol. 144, #3(1964):136-142.

67. McKercher, D. G., Wada, E. M. and Straub, Otto C.; Distribution and Persistence of Infectious Bovine Rhinotracheitis virus in Experimentally Infected Cattle. Am. J. Vet. Res., 24(1963): 510-514.

68. McKercher, D. G., Wada, E. M. and Straub, O.; Current Status of the Newer Virus Diseases of Cattle. Proc. 62nd Ann. Meeting U.S.L.S.A., 1957(1958):136-156.

69. Mclaren, Leroy C., Holland, John J. and Syverton, Jerome; The Mammalian Cell-Virus Relationships. I. Attachment of Poliovirus to Cultivated Cells of Primate and Non-primate Origin. J.Exp.Med. 109(1959):475-485.

70. Mims, C. A.; Aspects of the Pathogenesis of Virus Diseases. Bact. Rev., vol. 28(1964):30-71.

71. Mitchell, D.; An Outbreak of Abortion in a Dairy Herd Following Inoculation with an Intramuscular Infectious Bovine Rhinotracheitis virus Vaccine. Can. Vet. Jour., vol. 15(1974): 148-151.

72. Miyamoto, Kaneatsu and Morgan, Councilman; Structure and Development of Viruses as Observed in the Electron Microscope. XI. Entry and Uncoating of Herpes simplex virus. J. Virol., vol. 8(1971): 910-918.

73. Morgan, Councilman, Rose, Harry M. and Mednis, Baiba; Electron Microscopy of Herpes simplex virus. I. Entry. J. Virol., vol. 2(1968):507-516.

74. Nahmias, André J., Kibrick, Sidney and Rosan, Robert C.; Viral-Leukocyte Inter-relationships: I. Multiplication of a DNA virus-Herpes simplex, in Human Leukocyte Cultures. J. Imm. 93(1964): 67-74.

75. Oldendorf, William H.; Blood Brain Barrier Permeability to Drugs. Pharm. Rev. (1974):239-248.

76. Ole, Aalund, Hoerlein, A. B. and Adler, H. C.; The Migration Test on Circulating Bovine Leukocytes and its Possible Application in the Diagnosis of Johne's Disease. Acta. Vet. Scand., vol. 11 (1970):331-334.

77. Orgston; On the Uncertainties Inherent in the Determination of the Efficiency of Collisions Between Virus Particles and Cells. Biochim. Biophys. Acta. 66(1963):279.

78. Owens, N. V., Chow, T. L., and Molello, J. A.; Bovine Fetal Lesions Experimentally Produced by Bovine Infectious Rhinotracheitis virus. Am. J. Vet. Res. 25(1964):1617-1625.

79. Padawer, Jacques; Pox virus Phagocytosis in Vivo: Electron Microscopy of Macrophages, Mast Cells and Leukocytes. RES. J. Reti. Soc. 9(1971):23-41.

80. Perper, R. J., Zee, T. W. and Mickelson, M. M.; Purification of Lymphocytes and Platelets by Gradient Centrifugation. J. Lab. Clin. Med., 73(1968):842-848.

81. Rabinowitz, Yale; Separation of Lymphocytes, Polymorphonuclear Leukocytes and Monocytes on Glass columns, including Tissue culture Observations. Blood 23(1964):811-828.

82. Remington, Richard D. and Shock, M. Anthony; Statistics with Applications to the Biological and Health Sciences. Englewood Cliffs, New Jersey: Prentice-Hall, Inc.;(1970):24-29.

83. Reynolds, E. S.; The use of Lead Citrate at High pH as an Electron Opaque Stain in Electron Microscopy. J. Cell Biol., vol. 17 (1963):208-213.

84. Rouse, Barry T. and Babiuk, Lorne A.; Defence Mechanisms Against Infectious Bovine Rhinotracheitis virus: Inhibition of Virus Infection by Murine Macrophages. Inf. and Imm., vol. 11, #3 (1975):505-511.

85. Rubin, H., Franklin, R. M. and Baluda, M.; Infection and Growth of Newcastle Disease virus (NDV) in Chick Embryo Lung Epithelium. Virol. 3(1955):587.

86. Silva, R. F., Amborski, R. L., White, M. R. and Amborski, G. F.; An Electron Microscopic Study of a Single Cycle Infection of Madin-Darby Bovine Kidney Cells by Infectious Bovine Rhinotracheitis virus. J. Comp. Path., vol. 83(1973):161-172.

87. Snedecor, George W. and Cochran, William G.; Statistical Methods. The Iowa State University Press, Ames, Iowa, U.S.A., 6th Ed., 5th Printing, 1972.

88. Spring, S. B., Roizman, B. and Schwartz, Jerome; Herpes simplex virus Products in Productive and Abortive Infection. II. Electron Microscopic and Immunological Evidence for Failure of Envelopment as a Cause of Abortive Infection. J. Virol., vol. 2(1968): 384-392.

89. Steel, Robert G. D. and Torrie, James H.; Principles and Procedures of Statistics; with special reference to the Biological Sciences. McGraw-Hill Book Company, Inc., New York, 1960.

90. Stein, Silvia, Todd, Paul and Mahoney, Judith; Infectivity of Herpes simplex virus Particles with and without Envelopes. Can. J. Microb., vol. 16(1970):953-957.

91. Stevens, J. G. and Cook, Margery L.; Restriction of Herpes simplex virus by Macrophages. An Analysis of Cell-Virus Interaction. J. Exp. Med., Vol. 133, (1971):19-38.

92. Stevens, J. G. and Groman, N. B.; Properties of Infectious Bovine Rhinotracheitis virus in a Quantitated Virus-Cell Culture System. Am. J. Vet. Res., vol. 24(1963):1158.

93. Stossel, Thomas P.; Phagocytosis (First of three Parts). New England J. Med., vol. 290(1974):717-723.

94. Stossel, Thomas; Phagocytosis (Second of three Parts). New England J. Med., vol. 290(1974):774-780.

95. Stossel, Thomas; Phagocytosis (Third of three Parts). New England J. Med., vol. 290(1974):833-839.

96. Studdert, M. J., Baker, C. A. V. and Swan, Melton; Infectious Pustular Vulvovaginitis virus Infection of Bulls. Amer. J. Vet. Res. , 25 (1964) : 303-314.

97. Summerville, R. G. and Macfarlane, P. S.; The Rapid Diagnosis of Virus Infections by Immunofluorescent Techniques Applied to Blood Leukocytes. Lancet, April 25(1964):911-912.

98. Thomson, A. E. R., Bull, Jennifer M. and Robinson, Mary A.; A Procedure for Separating Viable Lymphocytes from Human Blood and Some Studies on their Susceptibility to Hypotonic Shocks. Brit. J. Haemat., vol. 12(1966):433-446.

99. Thorsby, E. and Bratlie, A.; A Rapid Method for Preparation of Pure Lymphocyte Suspensions. Histocompartibility Testing. Williams and Wilkings Company, Baltimore, MD (1970):655-656.

100. Valentine, R. C. and Allison, A. C.; Virus Particle Adsorption: I. Theory of Adsorption and Experiments on the Attachment of Particles to Non-biological Surfaces. Biochimica et Biophysica Acta, vol. 34(1959):10-23.

101. Wheelock, E. Frederick and Toy, Stephen T.; Participation of Lymphocytes in Viral Infections. Adv. Imm., vol. 17(1973): 123-184.

102. Willems, Frank T. C., Melnick, Joseph L. and Rawls, William E.; Replication of Poliovirus in Phytohemagglutinin Stimulated Lymphocytes. Journ. Virol. 3(1969):451-457.

103. World Health Organisation, Geneva, Switzerland. International Program on Animal Virus Characterisation.

104. Yoffey, J. M. and Sullivan, Eugene R.; The Lymphatic Pathway from the Nose and Pharynx. The Dissemination of Nasally Instilled Vaccinia virus. J. Exp. Med., vol. 69(1939):133-141.

105. Yoshitaka, Mori and Lennet, Karl, translated by Klaus Küchemann; Electron Microscopic Atlas of Lymph Node Cytology and Pathology. Springer-Verlag, Berlin, 1969.

106. Youngner, J. S.; Virus Adsorption and Plaque Formation in Monclayer Cultures of Trypsin Dispersed Monkey Kidney. J. Imm., vol. 76 (1956):288-292.

107. Zee, Y. C.; Personal Communication: Current Knowledge on Viral Receptors to Herpes viruses. 1975.

108. Zee, Y. C. and Talens, L.; Entry of Infectious Bovine Rhinotracheitis virus into Cells. J. Gen, Virol., 11(1971):59-63.

109. Zee, Y. C. and Talens, L.; Electron Microscopic Studies on the Development of Infectious Bovine Rhinotracheitis virus in Bovine Kidney Cells. J. Gen. Virol., 17(1972):333-336.

110. Zwick and Gminder; Untersuchungen über den "Bläschenausschlag" (Exanthema Vesiculosum Coitale) der Rinder. Berl. Tierärztl. Wchnschr., 29(1913):637-640.

TABLE 1. Summary of in vitro interactions of different blood cells with bovine herpesvirus - 1.

ACTIVITY[a]	CELL TYPE				
	Red blood cells	Polymorphonuclear cells	Monocytes	Lymphocytes with PHA*	Lymphocytes without PHA
Phagocytosis	-	+	+	-	-
Adsorption	+[b]	+[c]	+	+	+
Presence of viral nucleocapsids	-	±	+	+	±
Viral release	-	-	+	+	±

a: - = no activity
+ = activity always observed
± = activity irregularly observed

b: very low adsorption rates observed in the present study.

c: Indirect evidence from electron microscopy.

* PHA -- Phytohemagglutinin (Difco, M-Form).

FIGURE I

Flow Diagram of the Procedure Used in Isolating Cellular Components of Bovine Blood

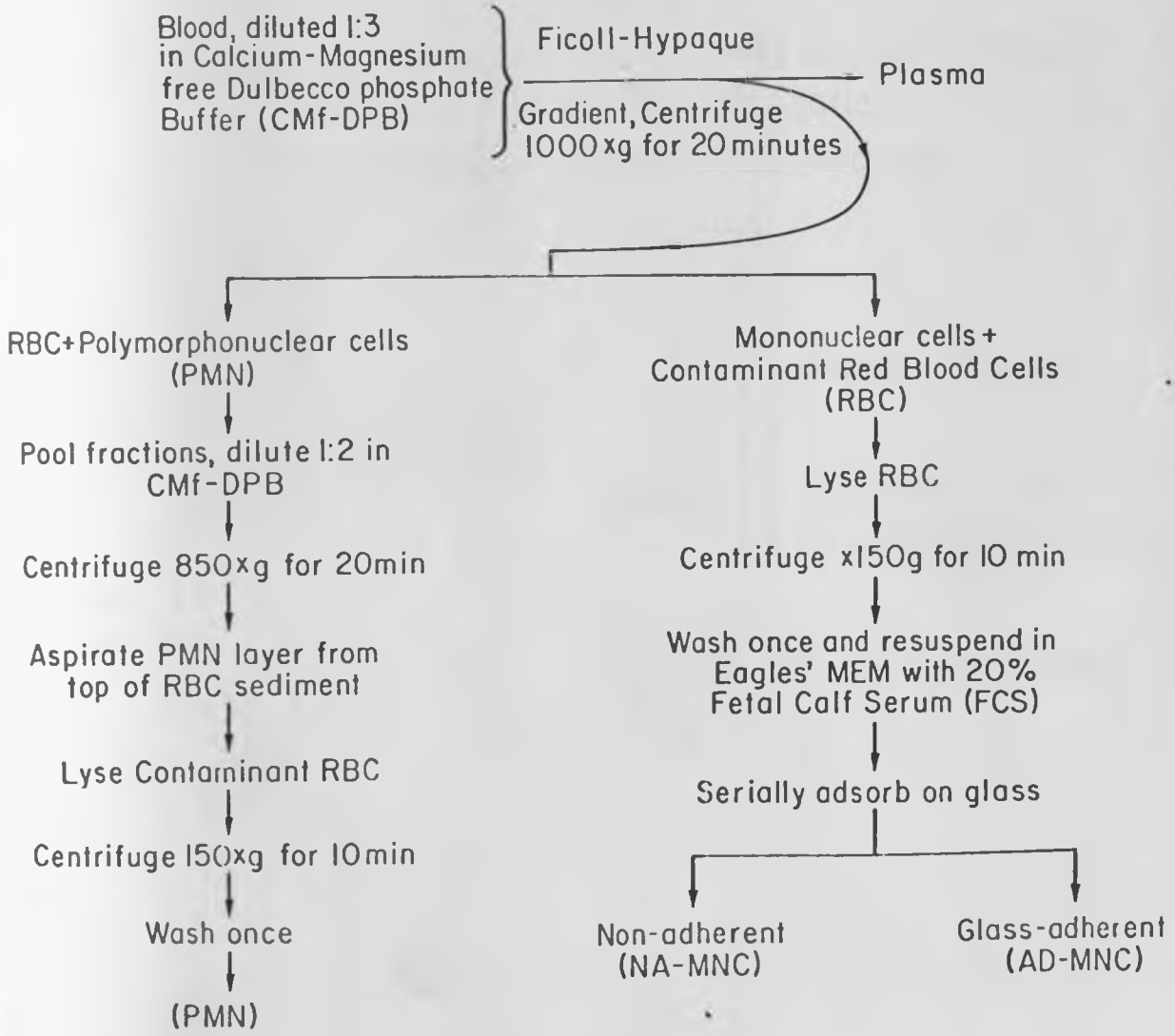


FIGURE 2

Separation of Cellular Components of Bovine Blood

(A) Before, (B) After Centrifugation

PMN's = Polymorhonuclear Leukocytes

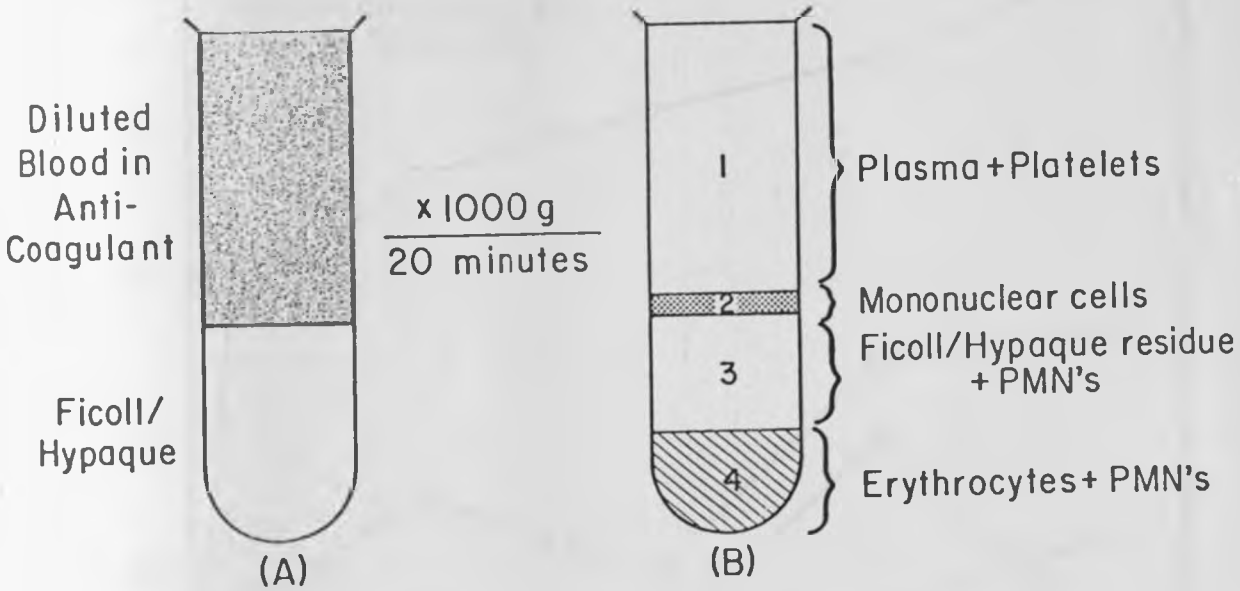


FIGURE 3

Adsorption of Bovine Herpes Virus-I (BHV-I) by Cells in Whole Blood and by Respective Separated Cellular Components

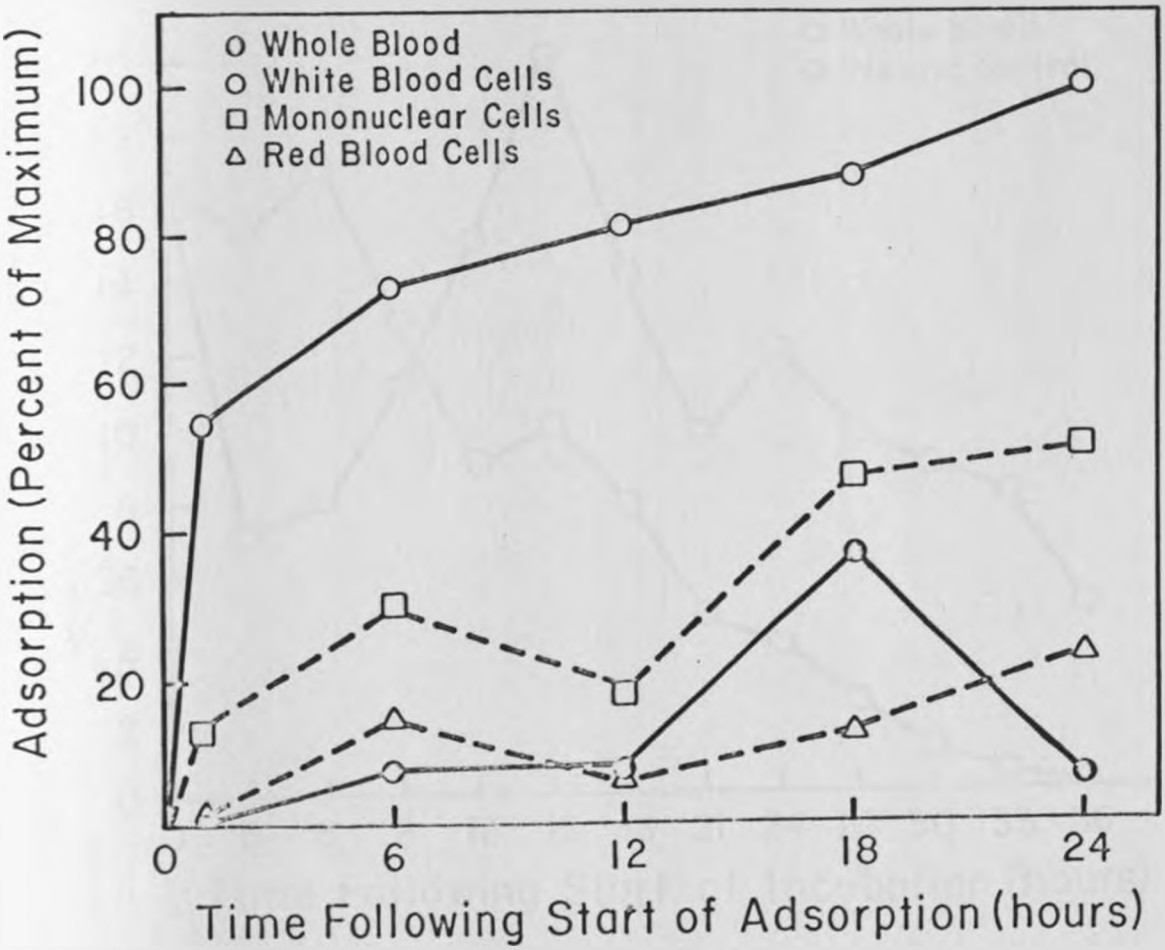


FIGURE 4

Replication of Bovine Herpes Virus-I (BHV-I) in Whole Bovine Blood

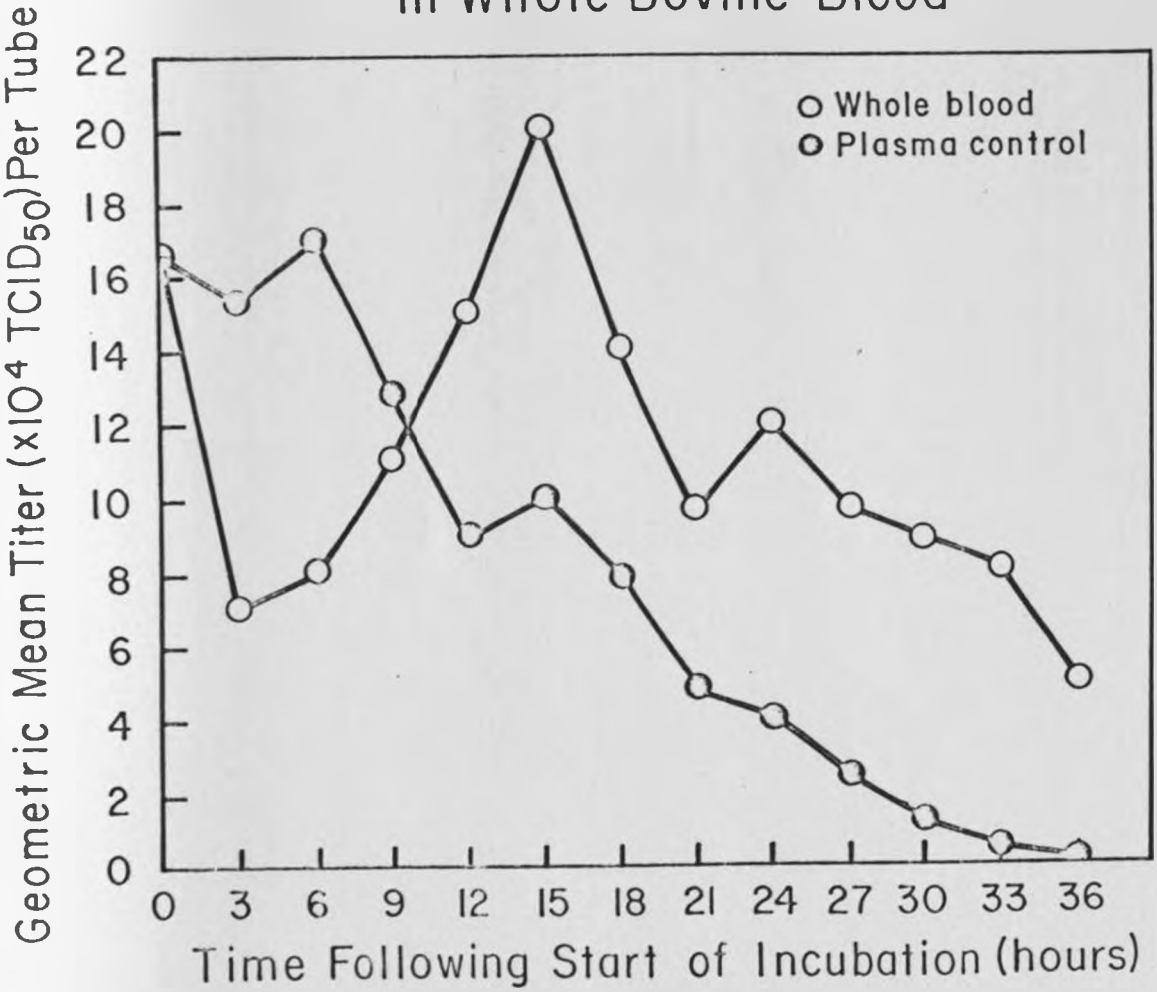


Figure 5

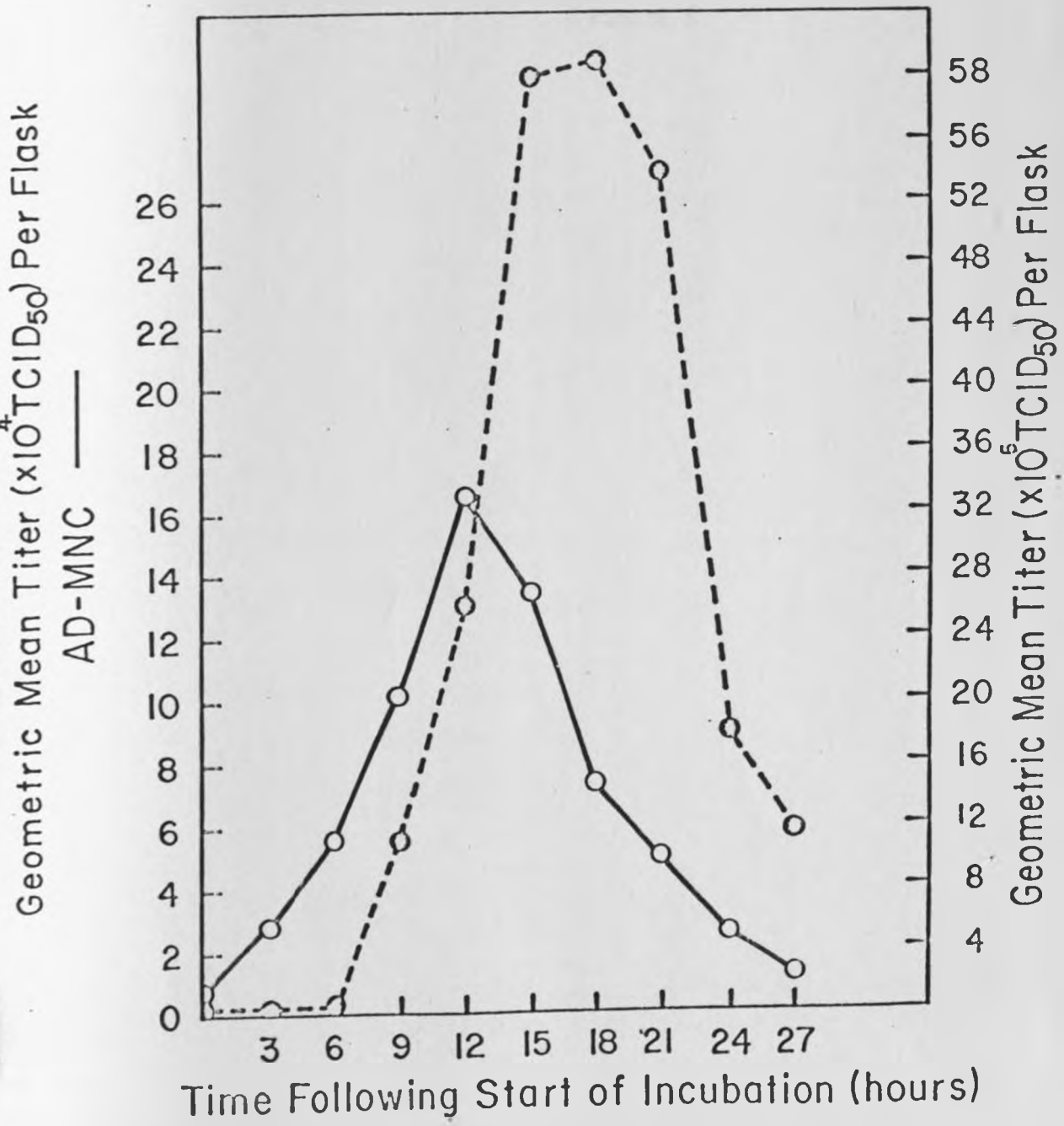
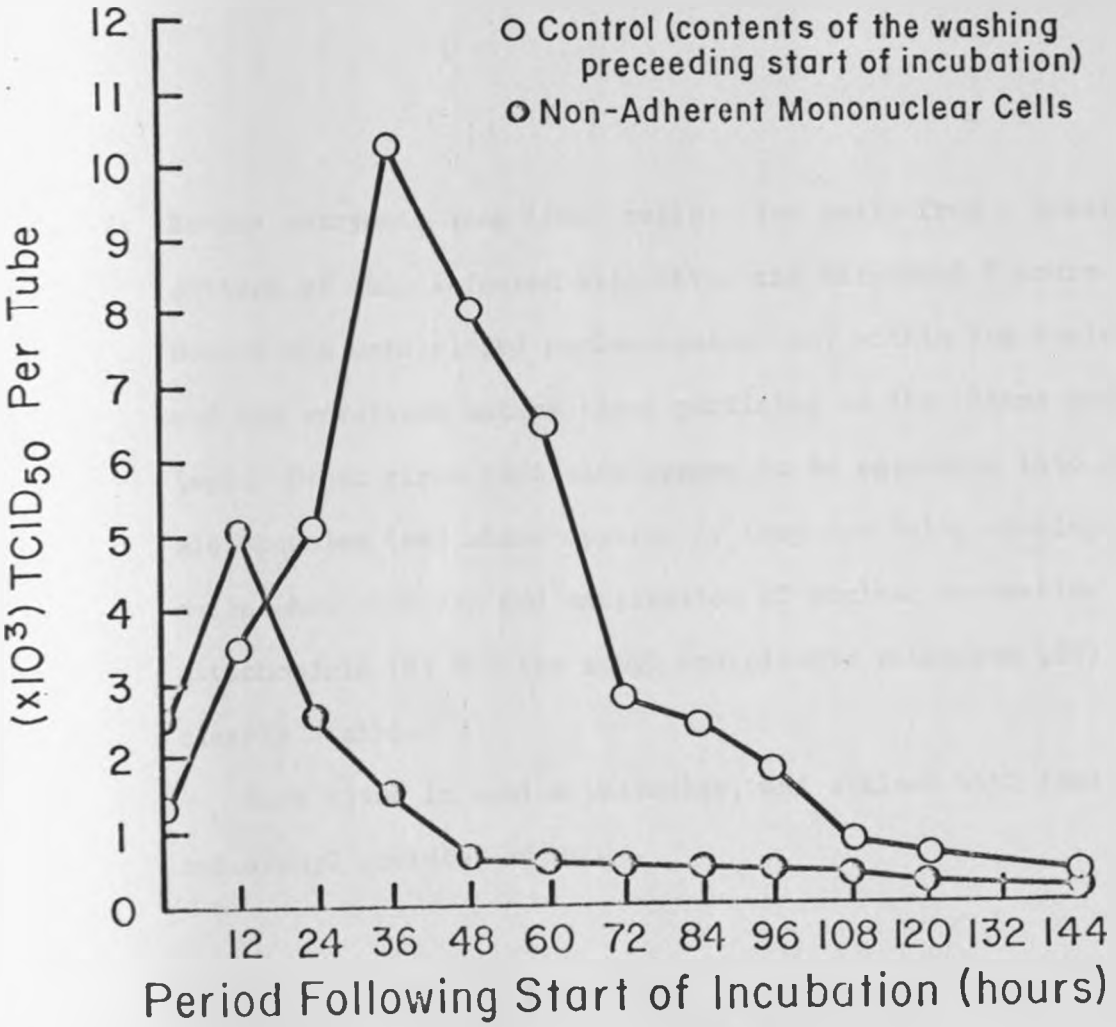

Geometric Mean Titer (x10⁴TCID50) Per Flask
AD-MNC ——
Geometric Mean Titer (x10⁵TCID50) Per Flask
0
2
4
6
8
10
12
14
16
18
20
22
24
26
4
8
12
16
20
24
28
32
36
40
44
48
52
56
58
3
6
9
12
15
18
21
24
27
Time Following Start of Incubation (hours)

Figure 6

The Replication of Bovine Herpes Virus-I (BHV-I) in Non-Adherent Mononuclear Cells



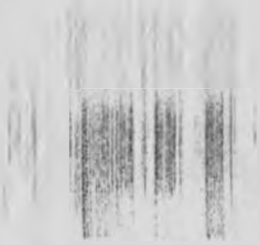


Fig. 7: Bovine embryonic lung (BEL) cells: Two cells from a monolayer culture of BEL, infected with BHV-1 and harvested 8 hours. Notice the unenveloped nucleocapsids (nc) within the nucleus (N) and the enveloped mature virus particles at the plasma membrane (mp). Other virus particles appear to be egressing into cytoplasmic vacuoles (cv) where apparently they are being enveloped. Both cells show clumping and margination of nuclear chromatin. The mitochondria (M) and the rough endoplasmic reticulum (ER) are clearly visible.

Post fixed in osmium tetroxide, and stained with lead citrate and uranyl acetate; x9,000.

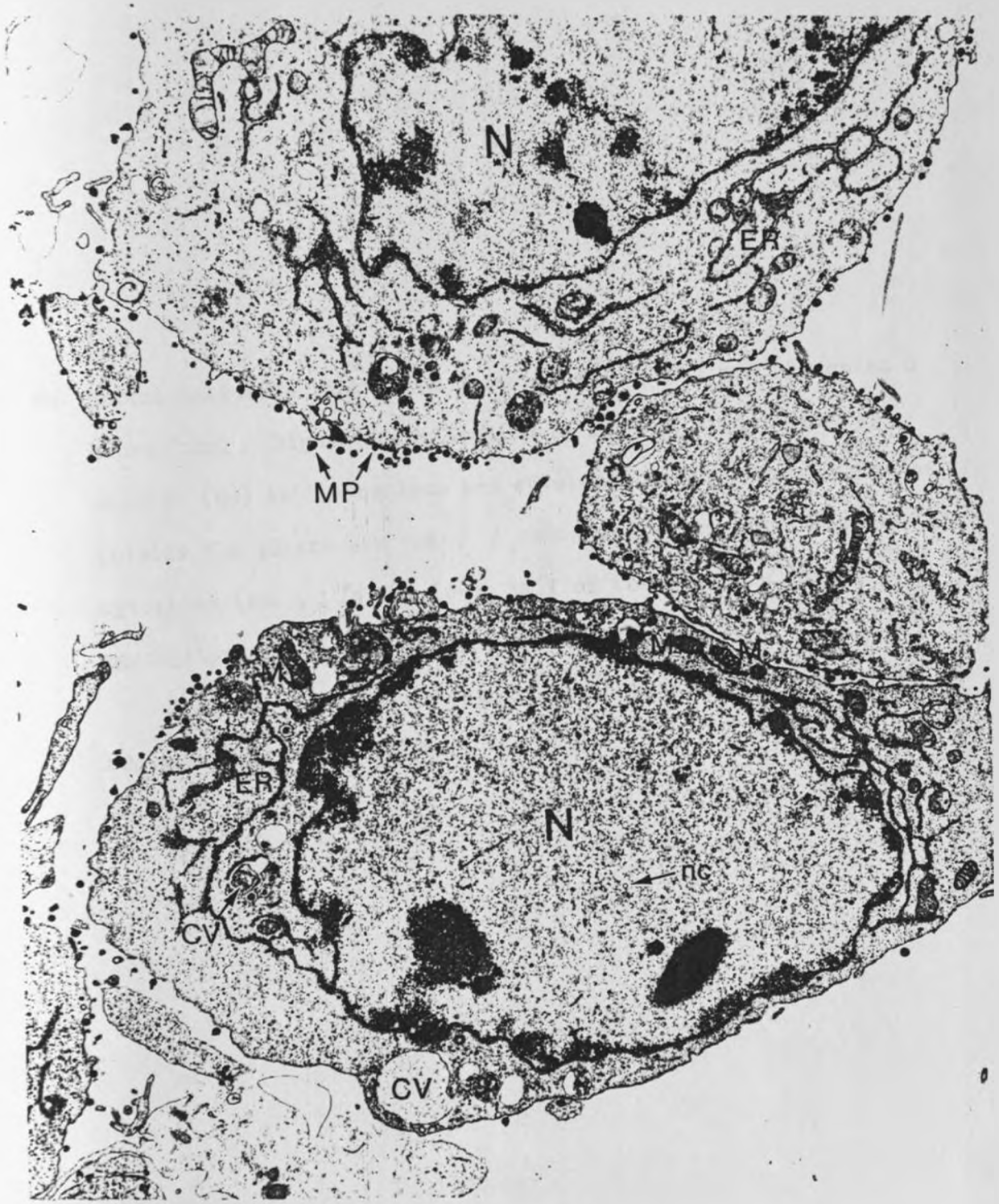

N
ER
MP
M
M
M
ER
N
nc
CV
CV

Fig. 8: Bovine Embryonic lung cells infected with EHV-1 and harvested 8 hours later. Higher magnification of fig. 7. Notice nucleocapsids (nc) in the nucleus and enveloped mature virus particles outside the plasma membrane. A naked nucleocapsid is seen in the cytoplasm (nnc). In the lower half of the cell are some aggregates of non-identifiable material (nim).

Post fixed in osmium tetroxide and stained with lead citrate and uranyl acetate; x73,000.

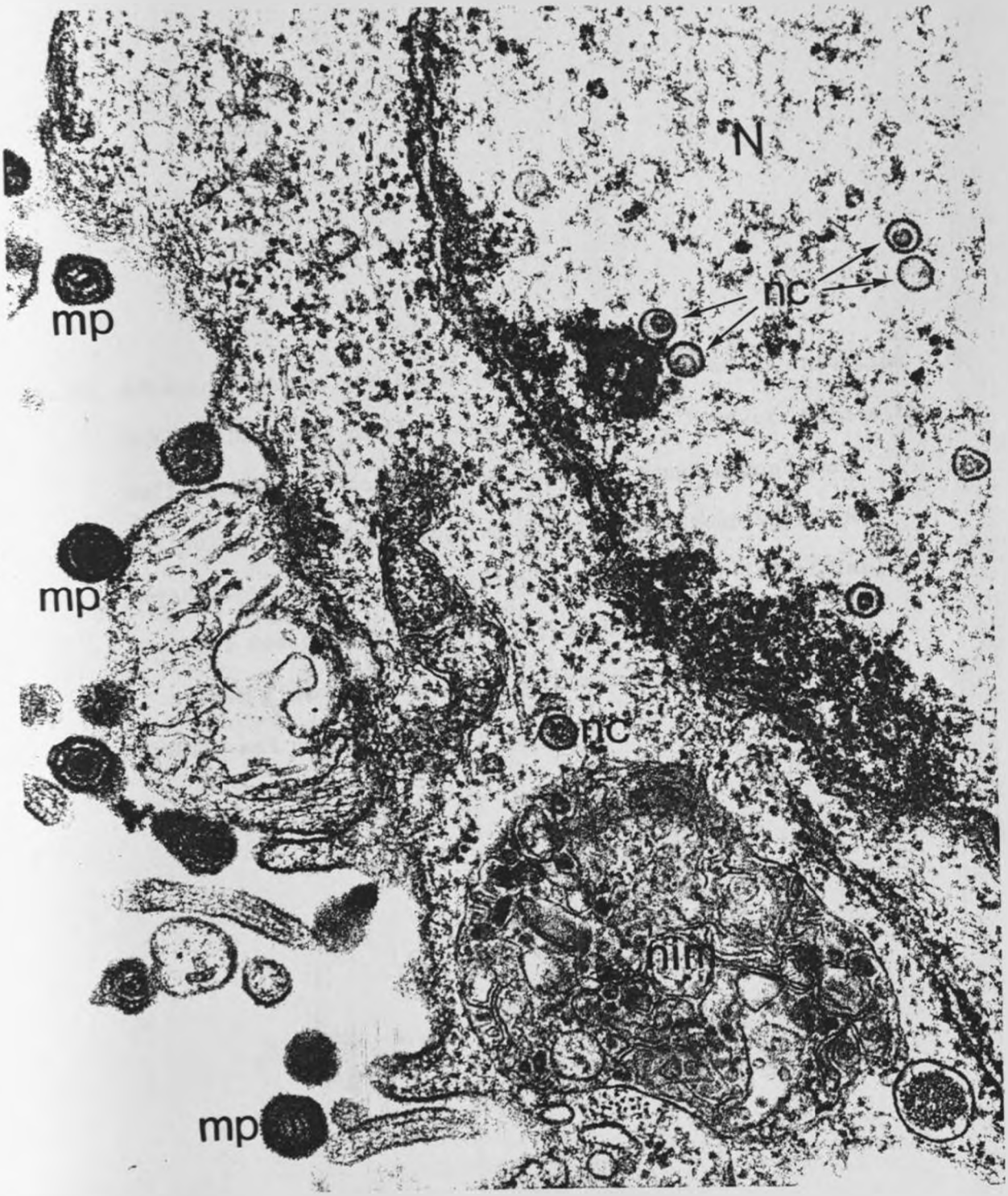

N
nc
mp
mp
nc
nm
mp

Fig. 9: Adherent Mononuclear cells harvested 5 hours after infection with BHV-1. No virus particles were observed. However, notice the well developed cytoplasmic organelles: rough endoplasmic reticulum (ER), Golgi apparatus (G), and mitochondria (M). Several poorly fixed mitochondria and some non-identifiable electron dense bodies (edb) are located in the cytoplasm.

Post fixed in osmium tetroxide and post stained in lead citrate and uranyl acetate; x9,000.

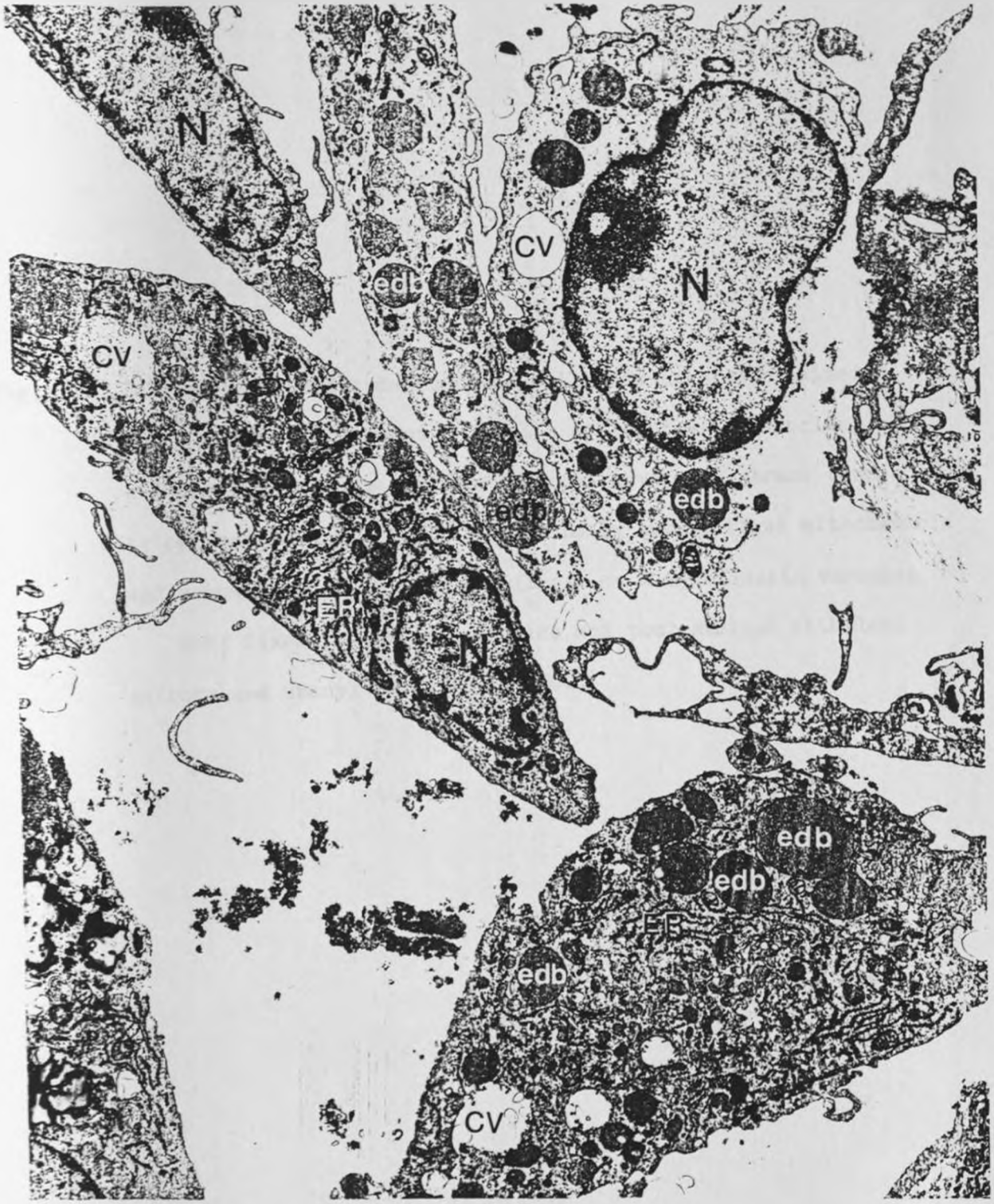

N
CV
edb
N
CV
edb
edb
ER
N
edb
edb
ER
edb
CV

Fig. 10: Part of an adherent mononuclear cell from samples obtained 8 hours after infection with BHV-1. Notice the intranuclear viral nucleocapsids in one nuclear lobe and several membrane bound cytoplasmic bodies. These are probably poorly fixed mitochondria and remnants of lysosomal granules; cv = cytoplasmic vacuoles.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x15,000.

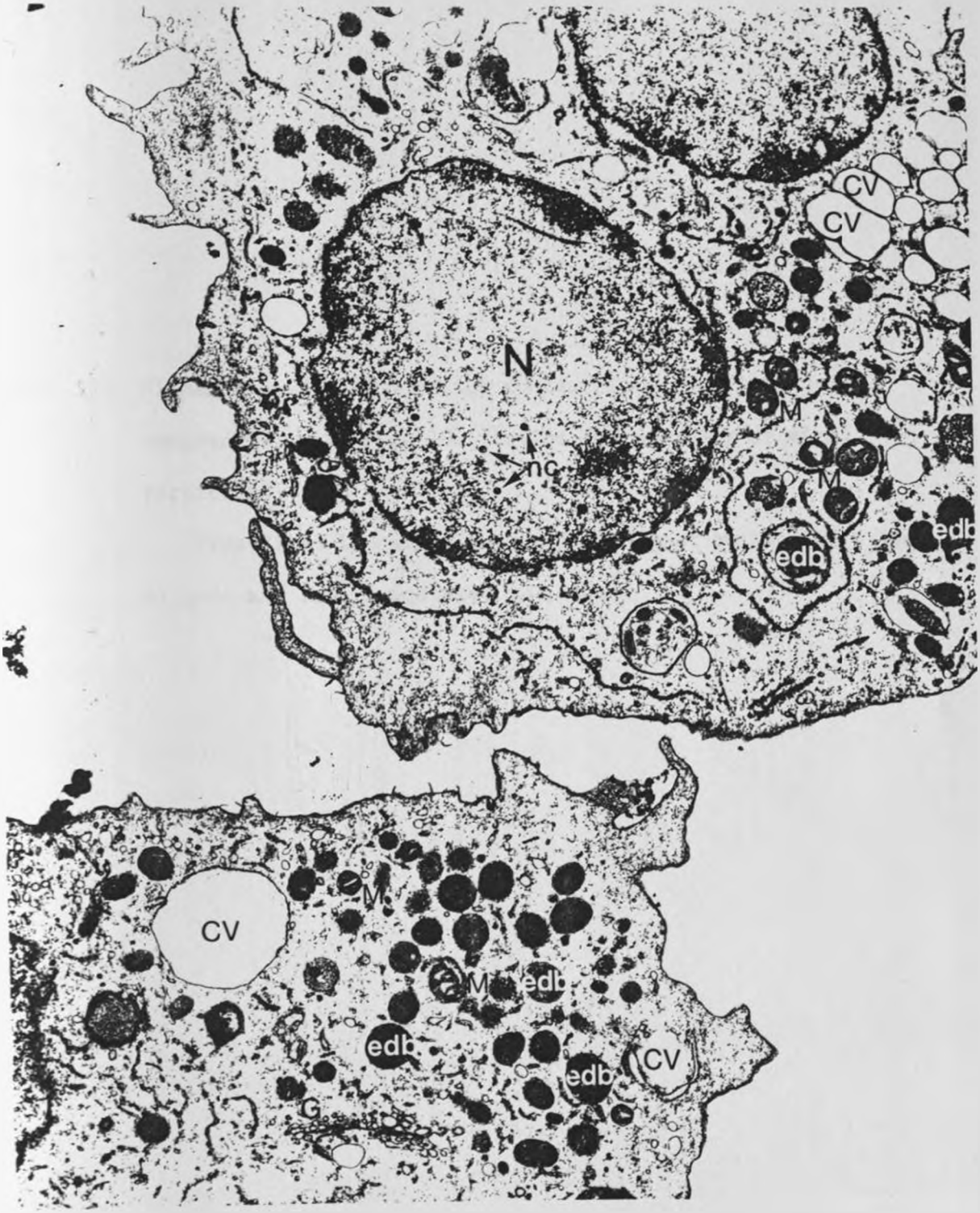

N
nc
CV
CV
M
M
edb
edb
CV
M
M
edb
edb
edb
CV
G

Fig. 11: Higher magnification of the nuclear lobe containing viral nucleocapsids in fig. 10. Notice the scarcity of viral particles.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x73,000.


nc
N
nc
nc
nc

Fig. 12: Part of an adherent mononuclear cell eighteen hours after infection with BHV-1. Notice the mature, enveloped virus particles (mp) located extracellularly close to the cell membrane. Some of the virus particles appear to be losing their morphological integrity.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x73,000.

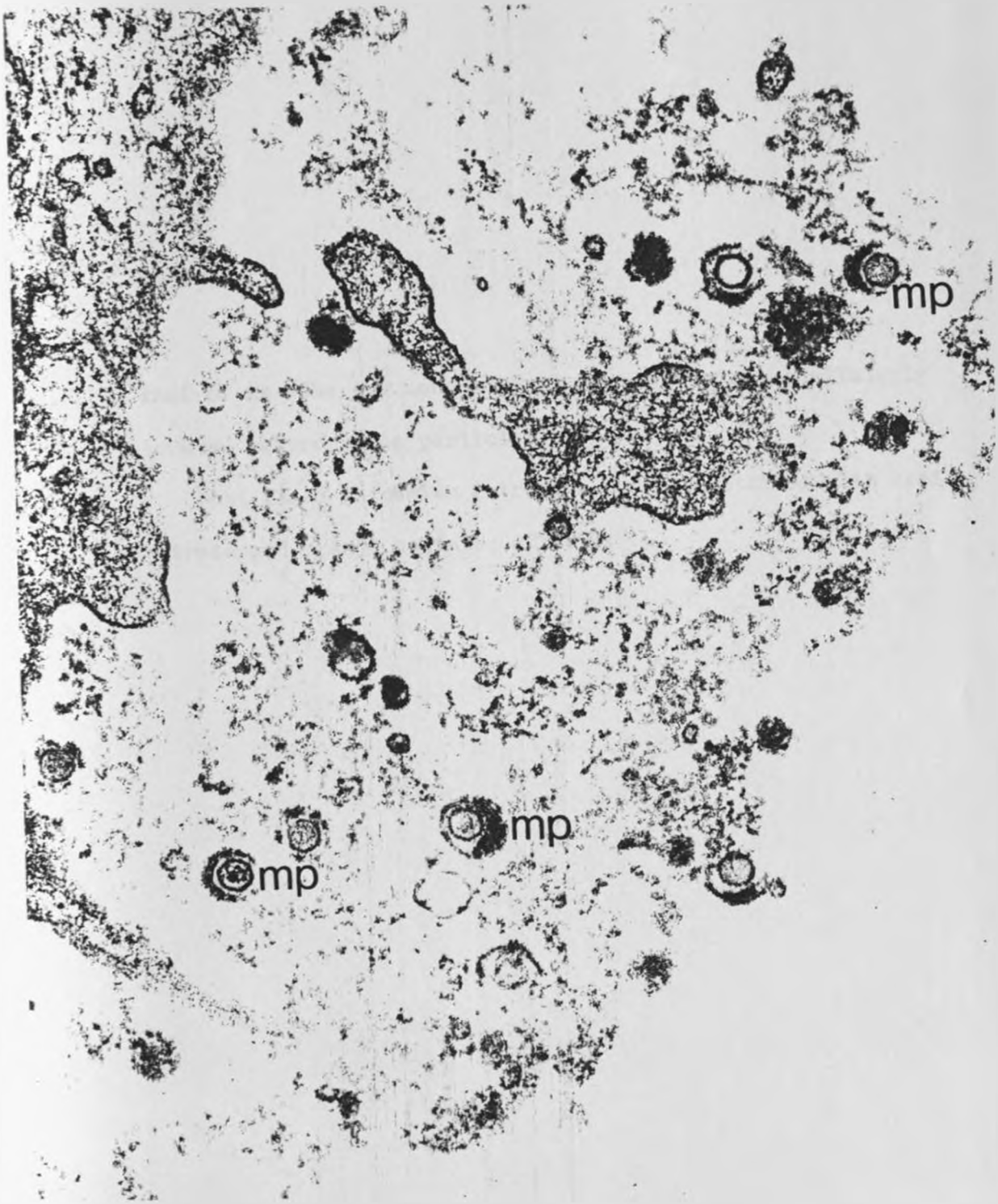

mp
mp
mp

Fig. 13: Part of an adherent mononuclear cell showing extracellularly located mature virus particles (mp).

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x73,000.

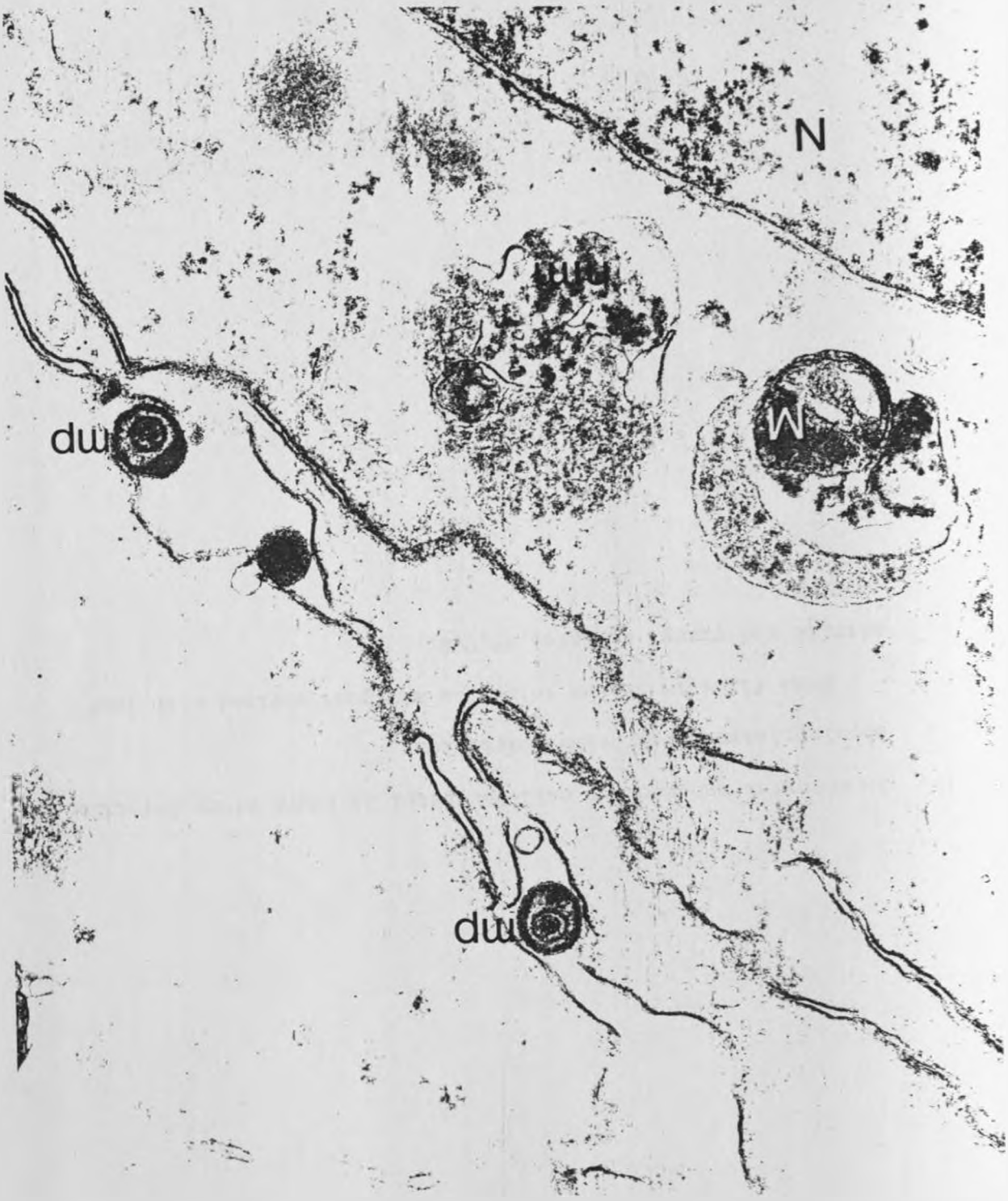

N
dw
dw
W
dw

Fig. 14: An adherent mononuclear cell harvested 14 hours after infection showing intranuclear viral particles.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x9,000.

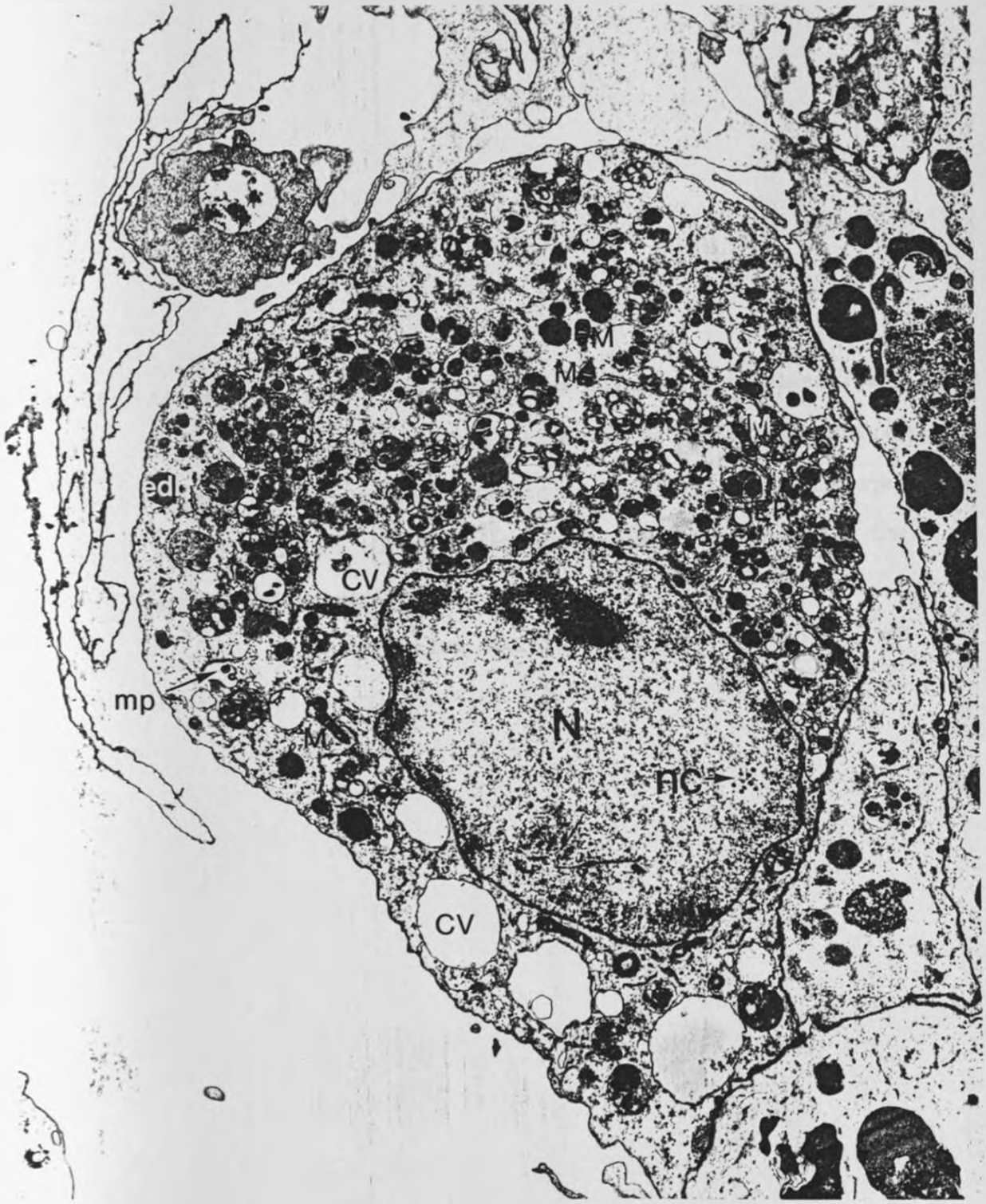

M
M
M
ed
CV
mp
M
N
nc
CV

Fig. 15: An adherent mononuclear cell from cultures maintained for 21 days in vitro. The cultures were infected with BHV-1 and harvested 8 hours later. No virus particles were seen in this preparation. However, notice the abundant protoplasmic processes and the cytoplasmic vesicles.

Post fixed in osmium tetroxide and post stained in lead citrate and uranyl acetate; x15,000.

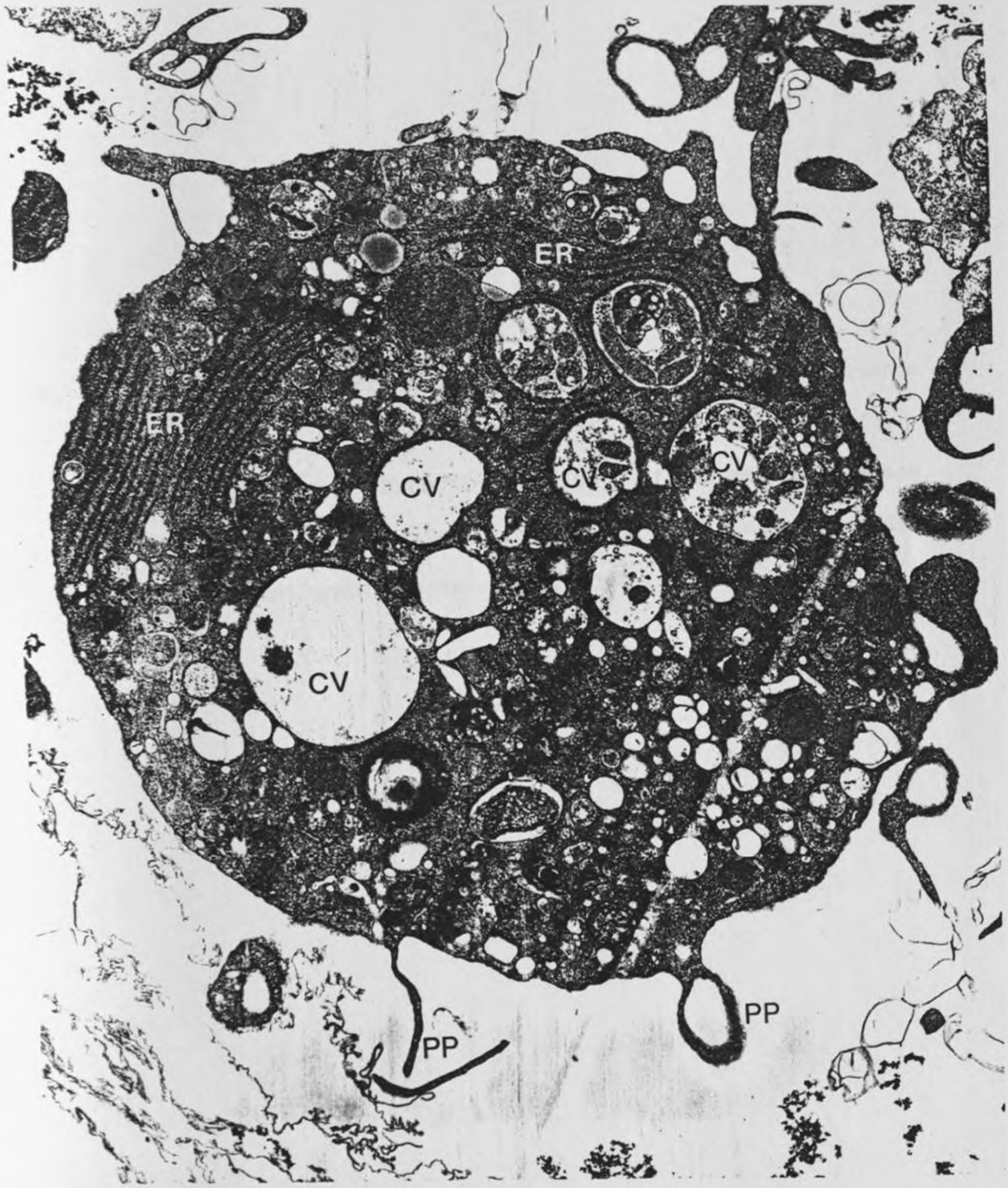

ER
ER
CV
CV
CV
CV
PP
PP

Fig. 16: A normal non-adherent mononuclear cell. Notice the presence of mitochondria close to the Golgi area in the nuclear cleft, and dense peripheral chromatin and clumps of central nucleoplasm.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x38,000.

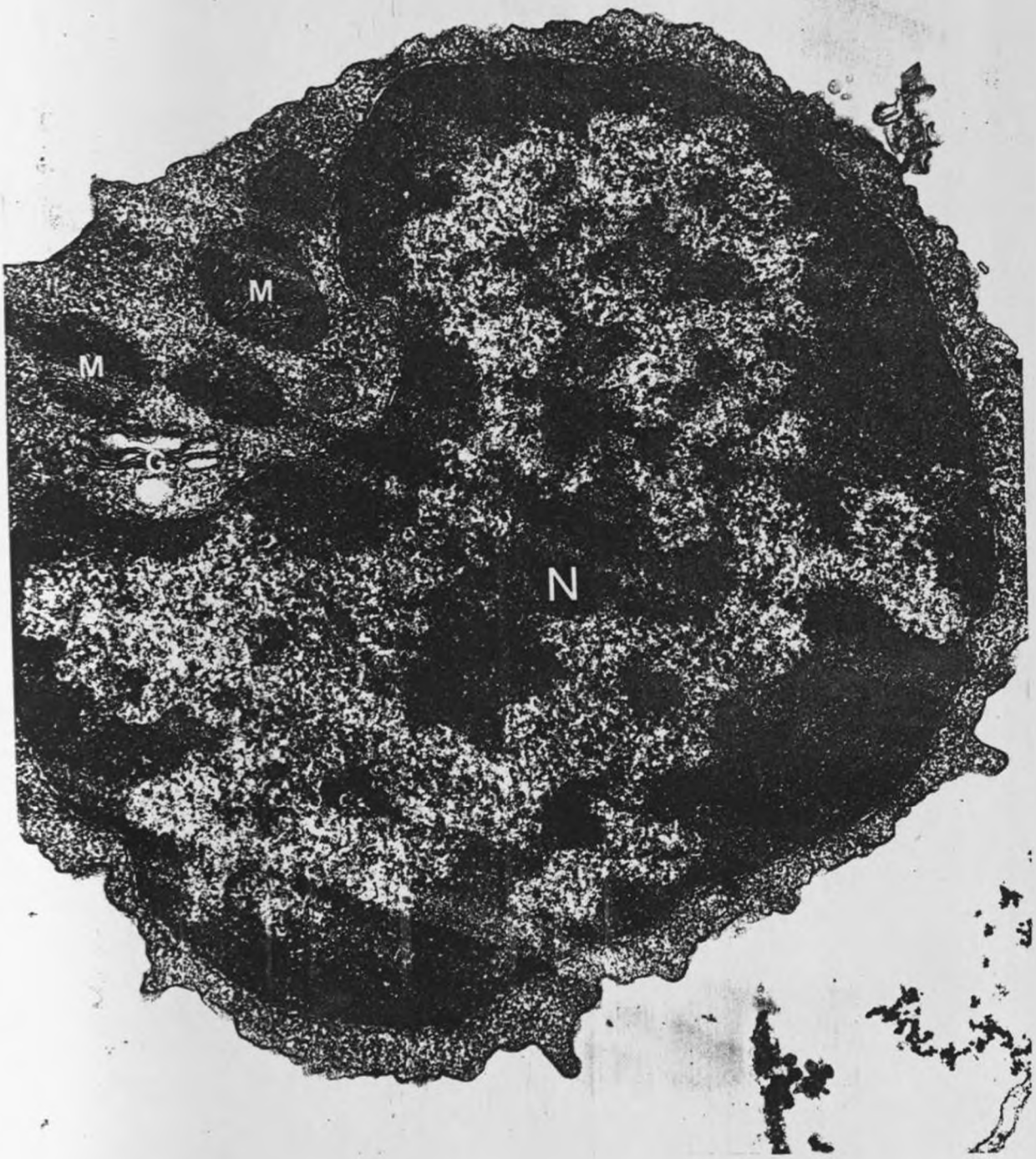

M
M
G
N

Fig. 17: Part of a non-adherent mononuclear cell from samples harvested 14 hours after infection with BHV-1.

Notice the virus particles in the nucleus only.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x73,000.

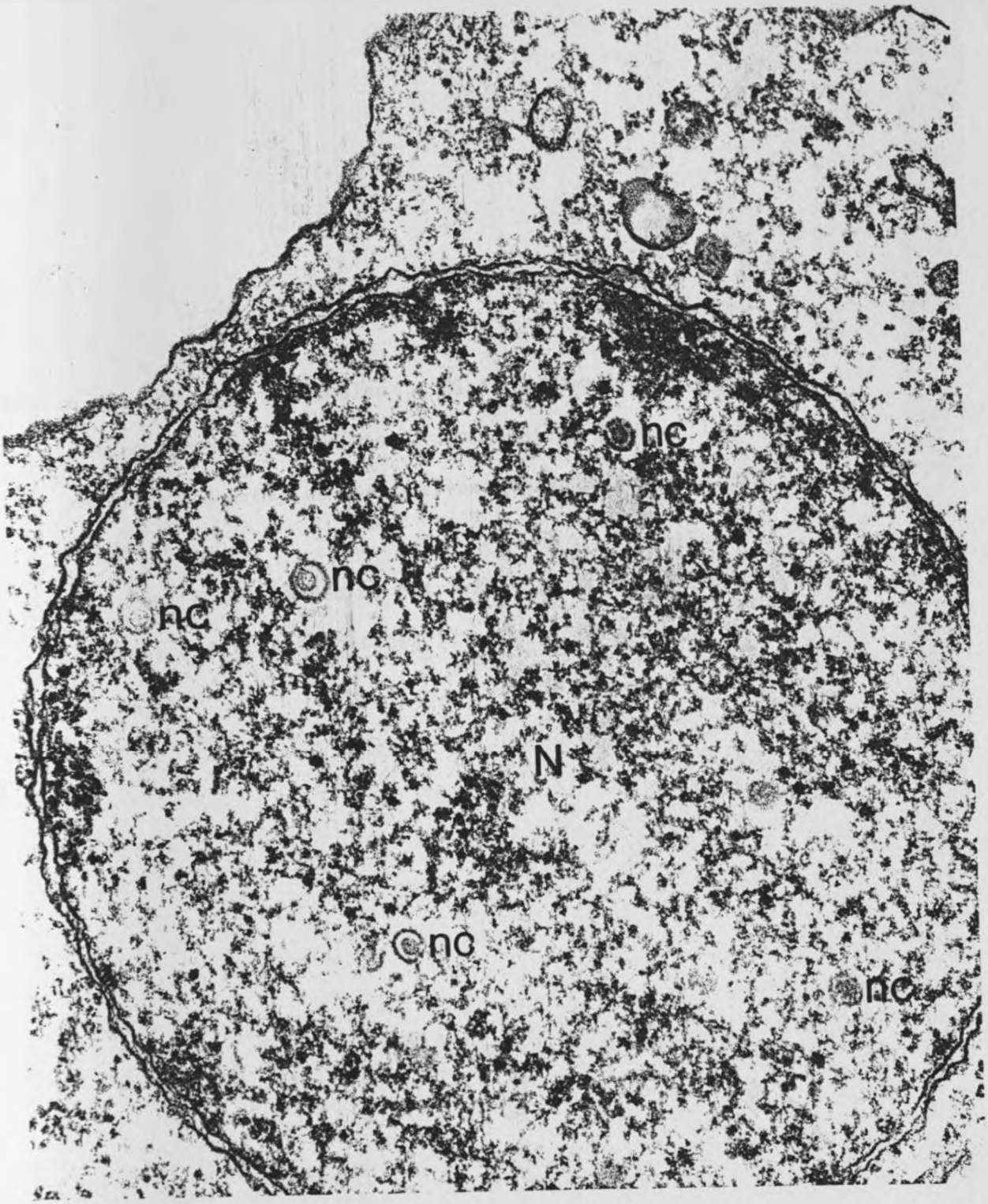

nc
nc
nc
N
nc
nc

Fig. 18: A non-adherent mononuclear cell from the 14 hour harvest. Notice the absence of viral particles in this cell, whereas other cells from the same harvest (fig. 17) showed viral particles.

Post fixed in osmium tetroxide, post stained with lead citrate and uranyl acetate; x19,000.

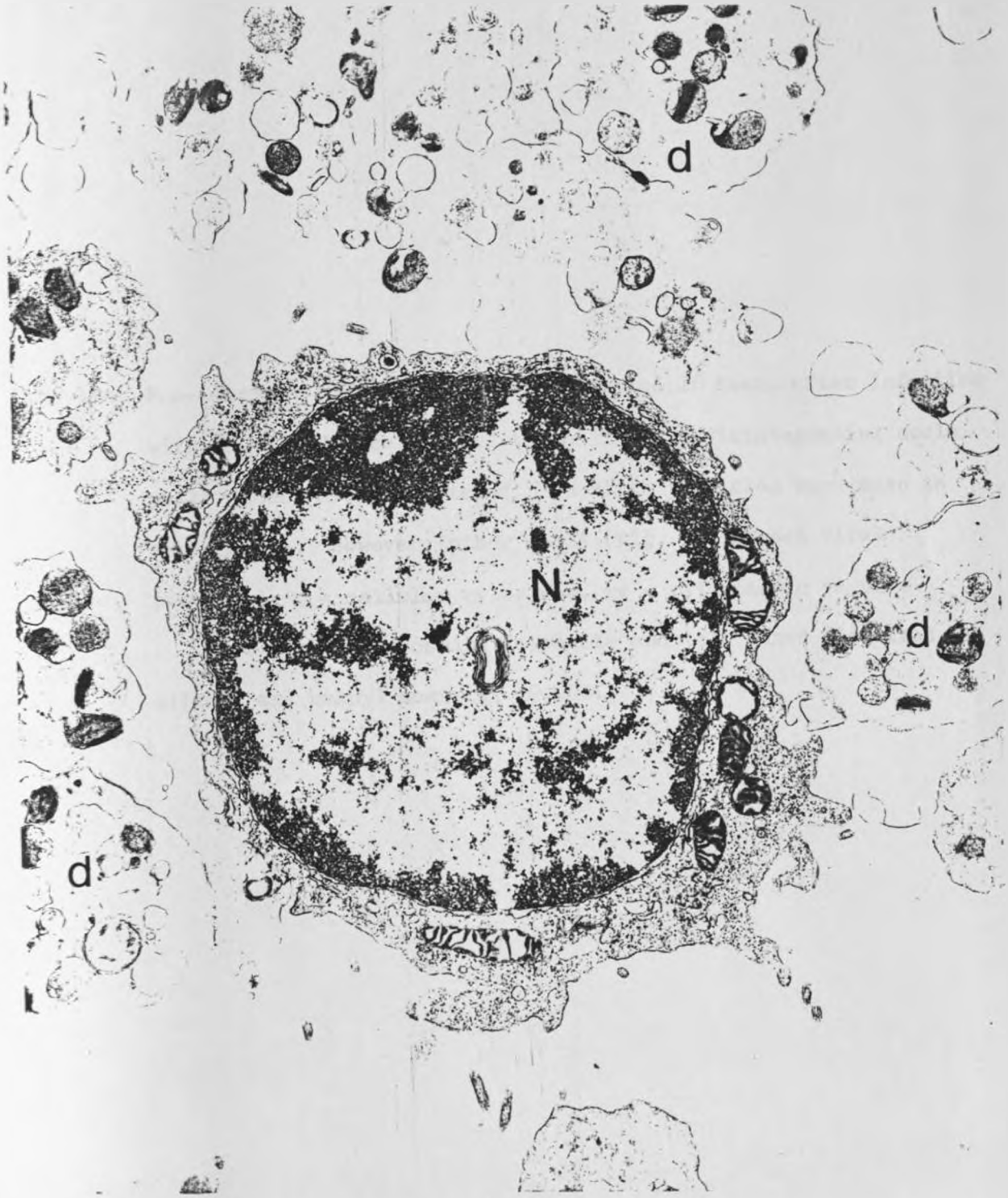

d
N
d
d

Fig. 19: Non-adherent mononuclear cells harvested 18 hours after infection with BHV-1. Notice some of the cells have disintegrating nuclei while others seem unaffected. No viral particles were seen in these cells. However, other cells (fig. 21) showed viral particles; c = cellular cytoplasm, cv = cytoplasmic vacuole.

Post stained in osmium tetroxide and post fixed with lead citrate and uranyl acetate; x9,000.

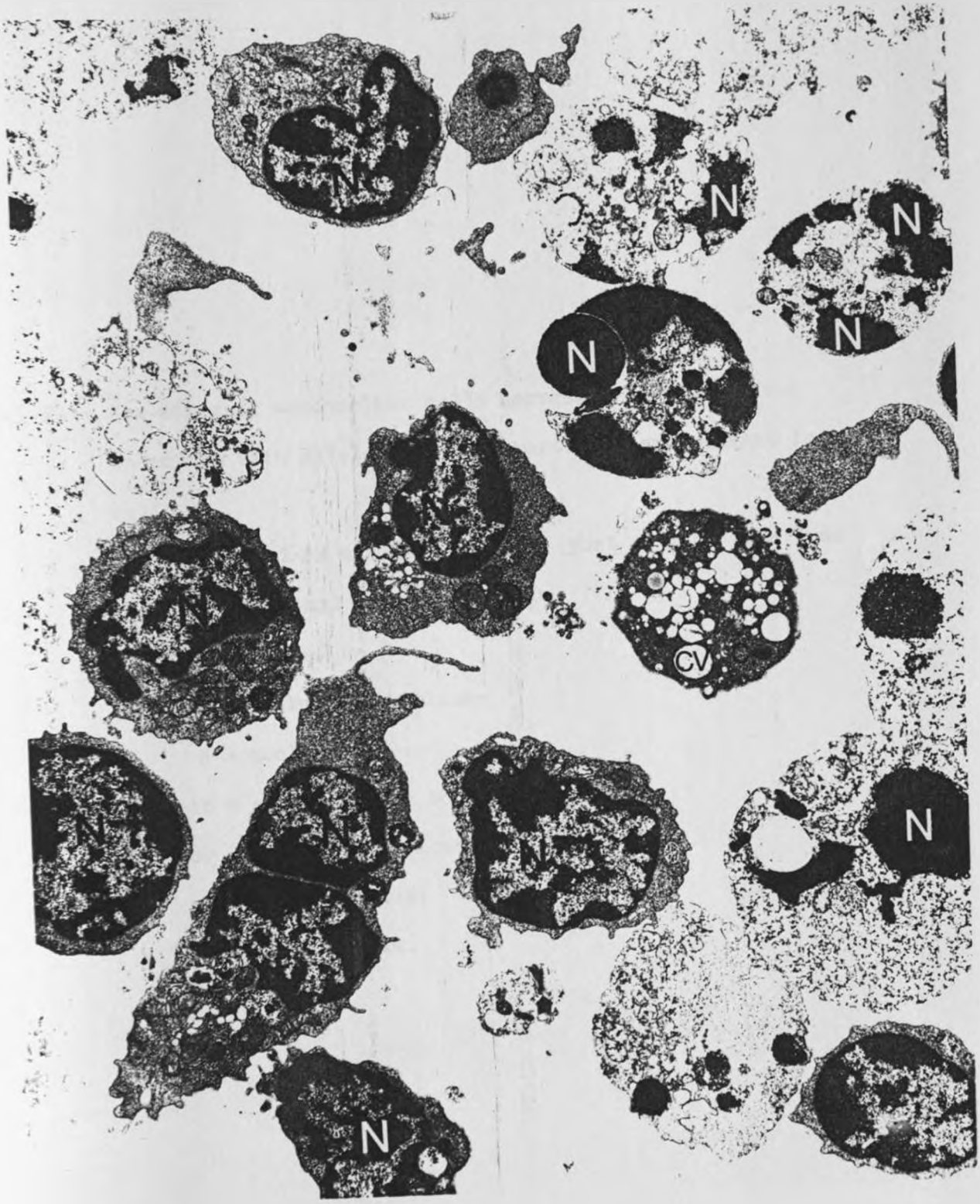

N
N
N
N
N
CV
N
N
N
N

Fig. 20: Non-adherent mononuclear cells harvested 18 hours after infection with BHV-1. No virus particles are present in these cells.

Post fixed in osmium tetroxide, post stained with lead citrate and uranyl acetate; x9,000;

nl = nucleolus;

c = cellular cytoplasm;

N = nucleus;

edb = electrondense body;

cv = cytoplasmic vacuole;

G = Golgi complex;

M = mitochondria.

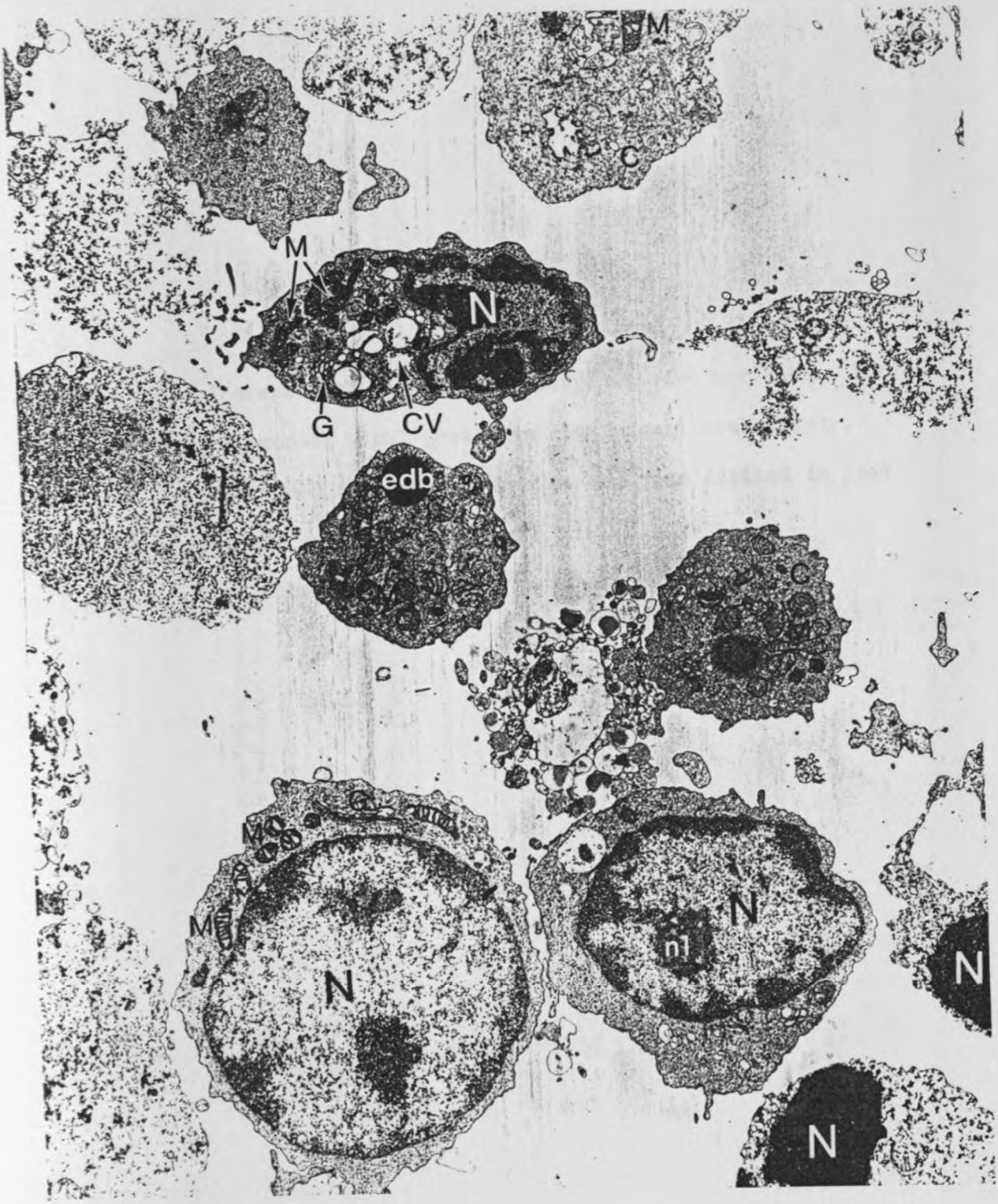

M
C
M
N
G
CV
edb
C
M
N
N
nl
N
N

Fig. 21: Non-adherent cell from 20 hour sampling time harvests. Notice the presence of viral particles located extracellularly.

Post fixed in osmium tetroxide and post stained in lead citrate and uranyl acetate; x73,000.

c = cellular cytoplasm

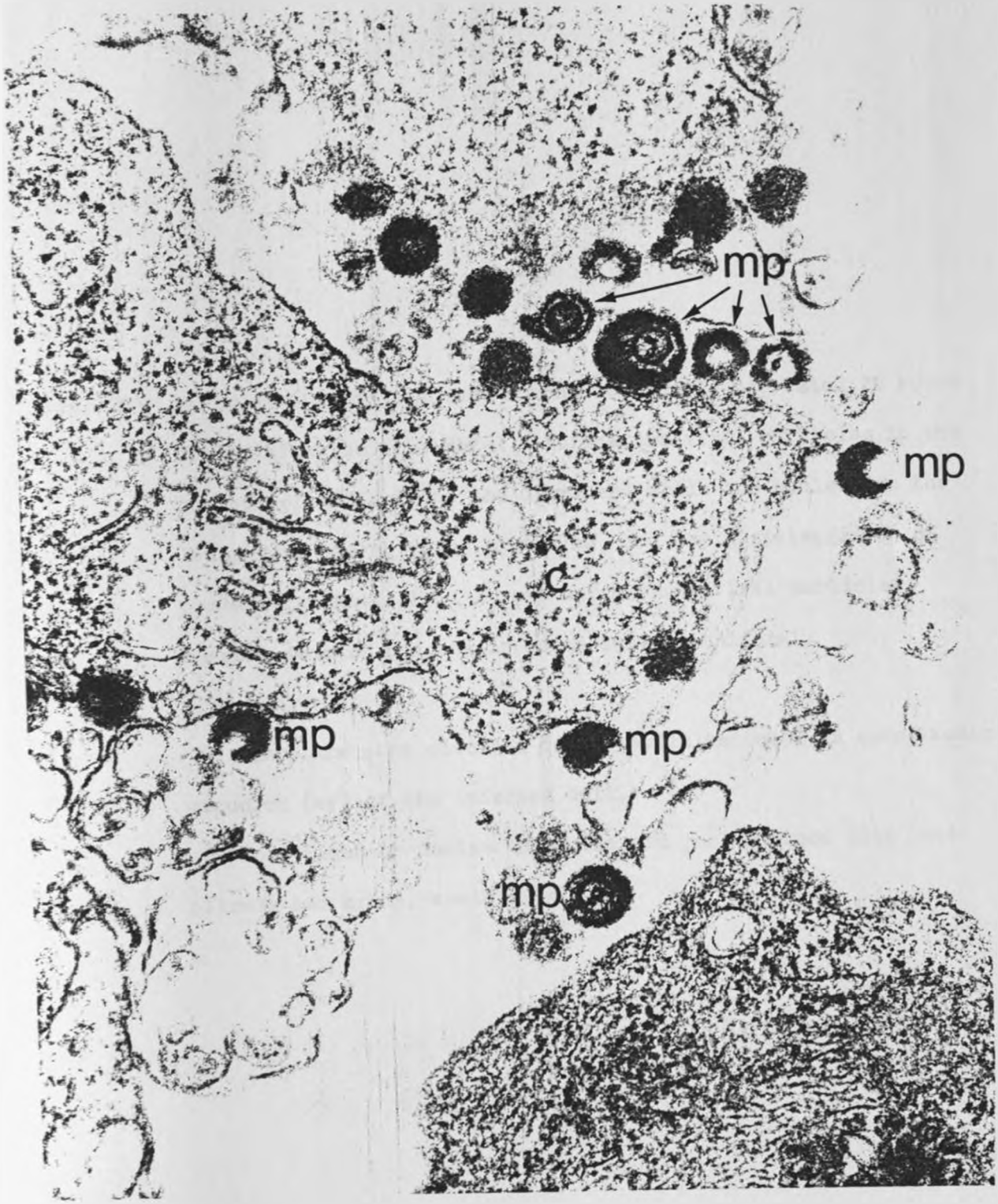

mp
mp
c
mp
mp
mp

Fig. 22: Parts of two non-adherent mononuclear cells harvested 20 hours after infection with BHV-1. Notice the viral particles in the nucleus of the cell at the upper end while the nucleus in the lower cell has no virus particles, and the disintegration of nuclear chromatin in the cell which shows viral particles whereas that of the non-infected cell is uniformly electronlucent.

There are some electron dense particles (edp) in cytoplasmic vacuoles (cv) of the infected cell.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x19,000.

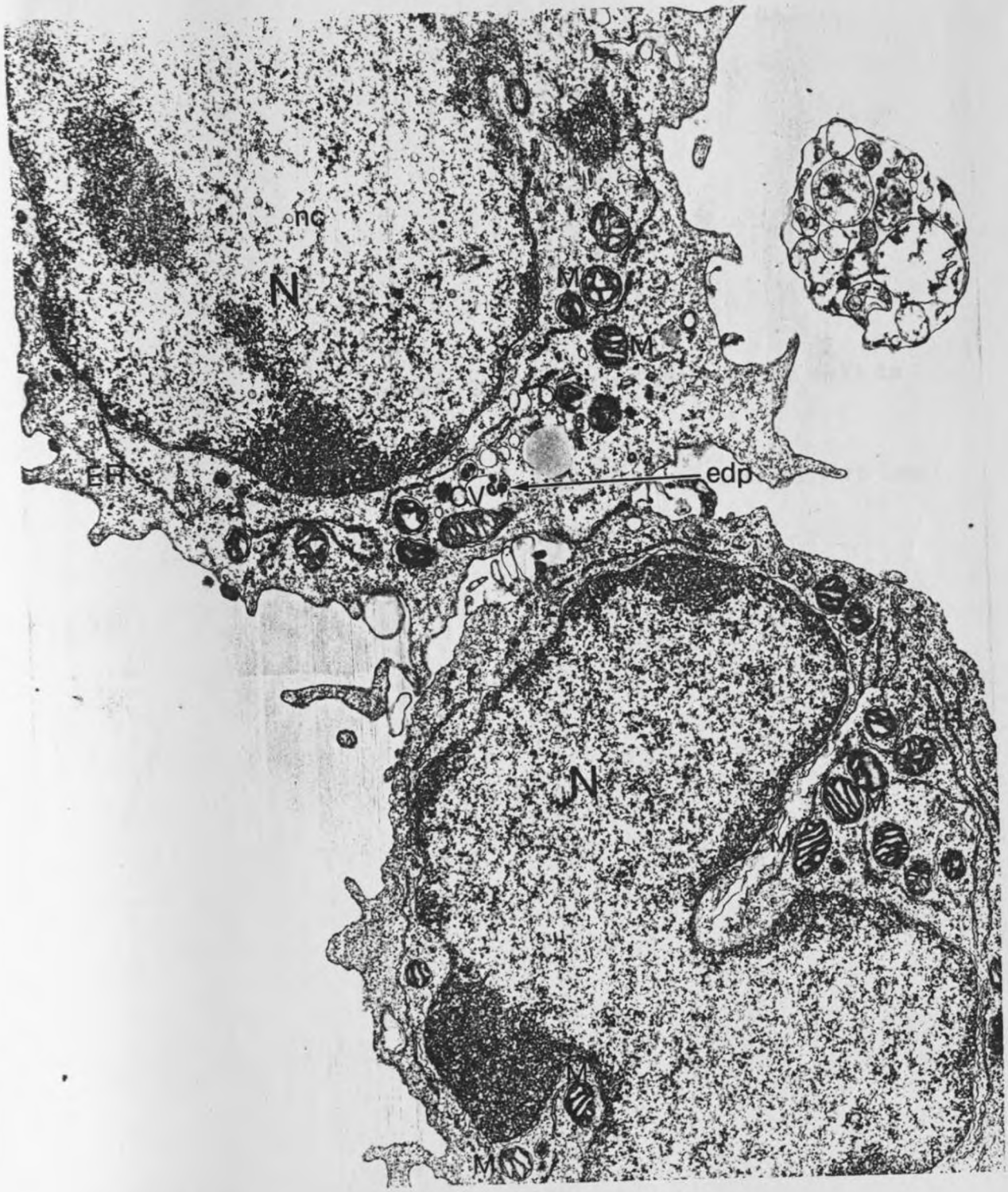

nc
N
M
M
ER
dv
edp
N
M
M

Fig. 23: Higher magnification of the nucleus of the infected cell in fig. 22.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x73,000.

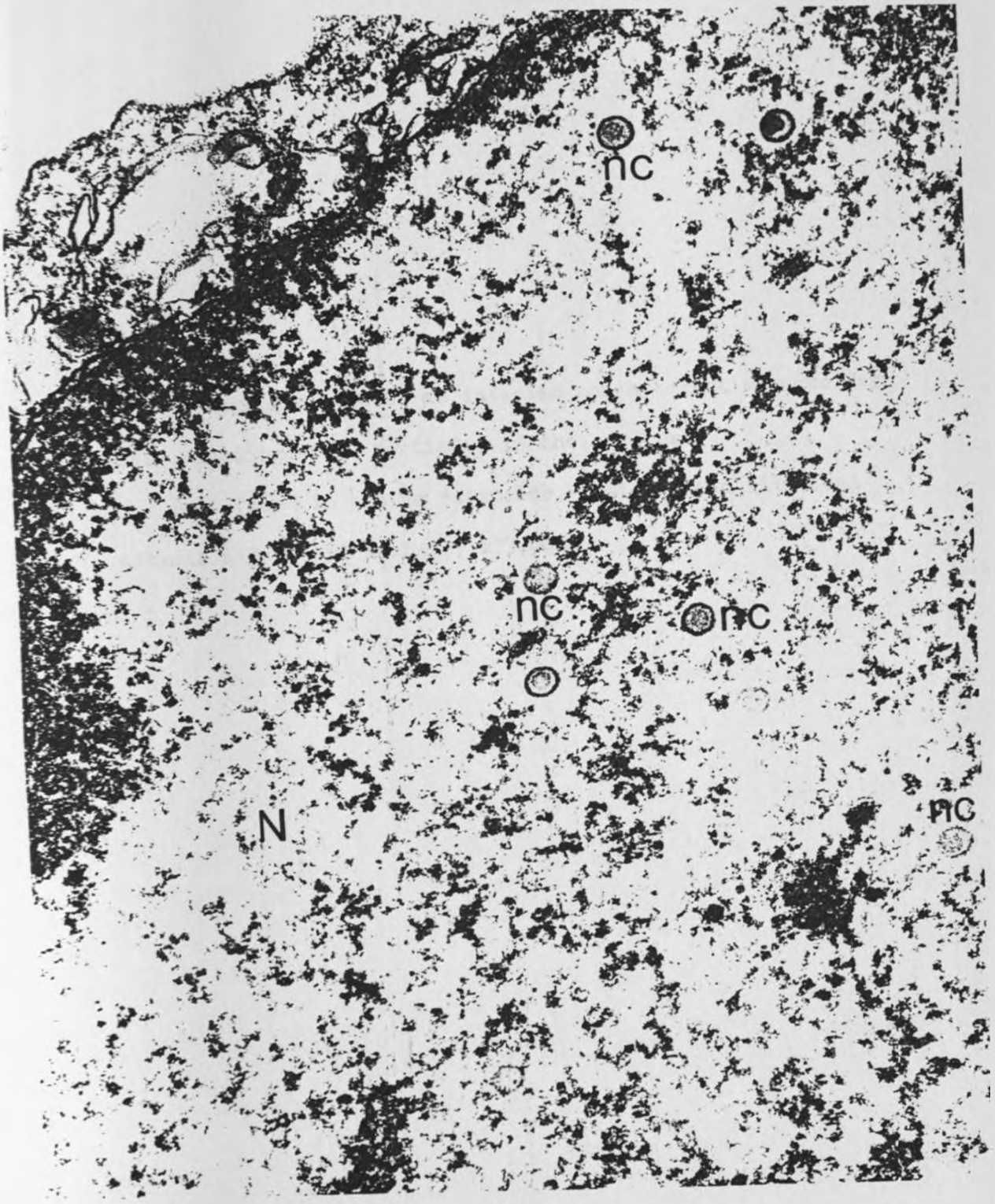

nc
nc
nc
N
nc

Fig. 24: A high magnification of an infected non-adherent mononuclear cell, showing viral particles in the nucleus.

Post fixed in osmium tetroxide, post stained with lead citrate and uranyl acetate; x73,000.

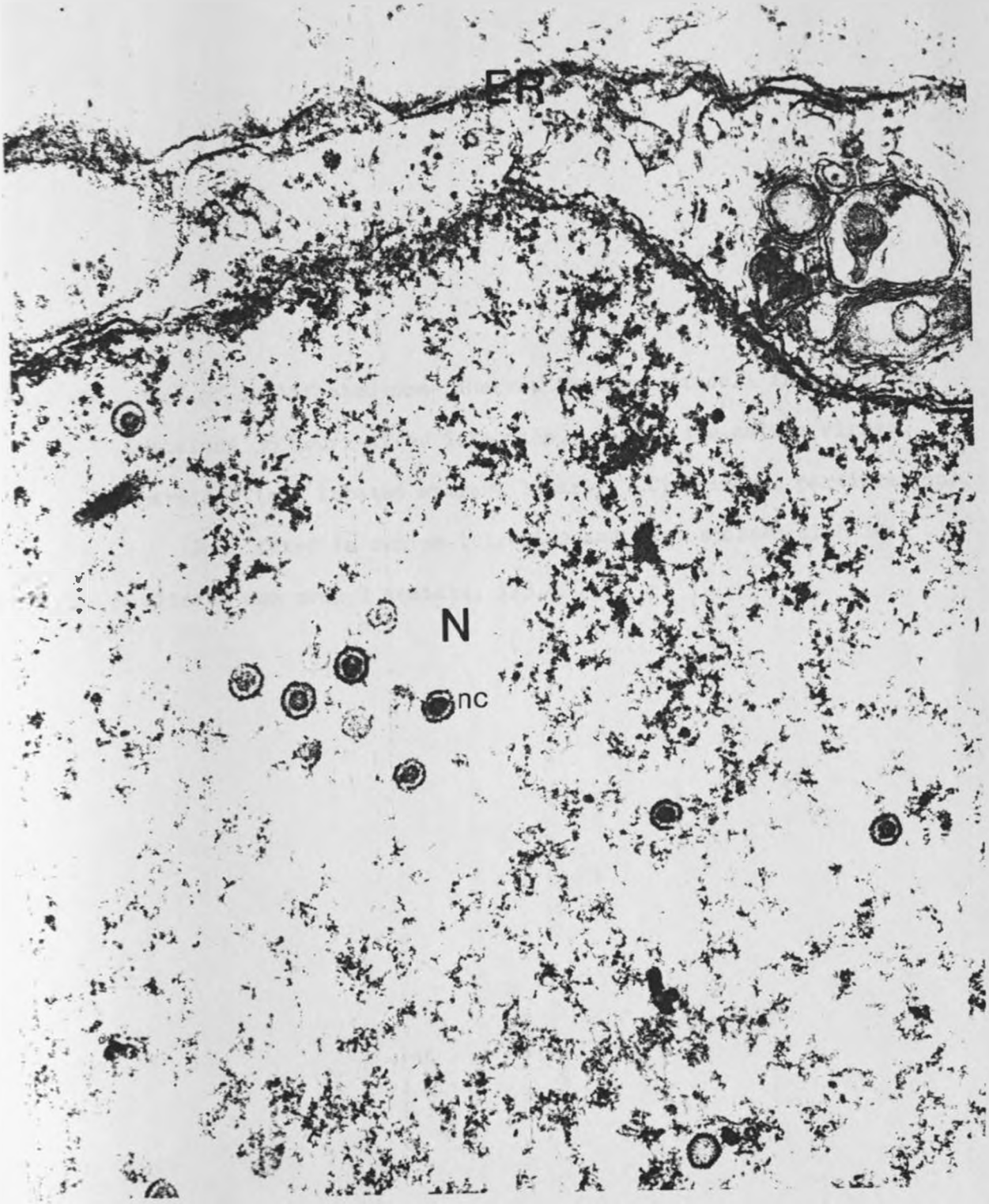

ER
N
nc

Fig. 25: Part of an infected non-adherent mononuclear cell from samples obtained 36 hours after infection. Notice the mature virus particle (mp) located extracellularly. Cytoplasmic particle (cp).

Post fixed in oxmium tetroxide and post stained with lead citrate and uranyl acetate; x73,000.

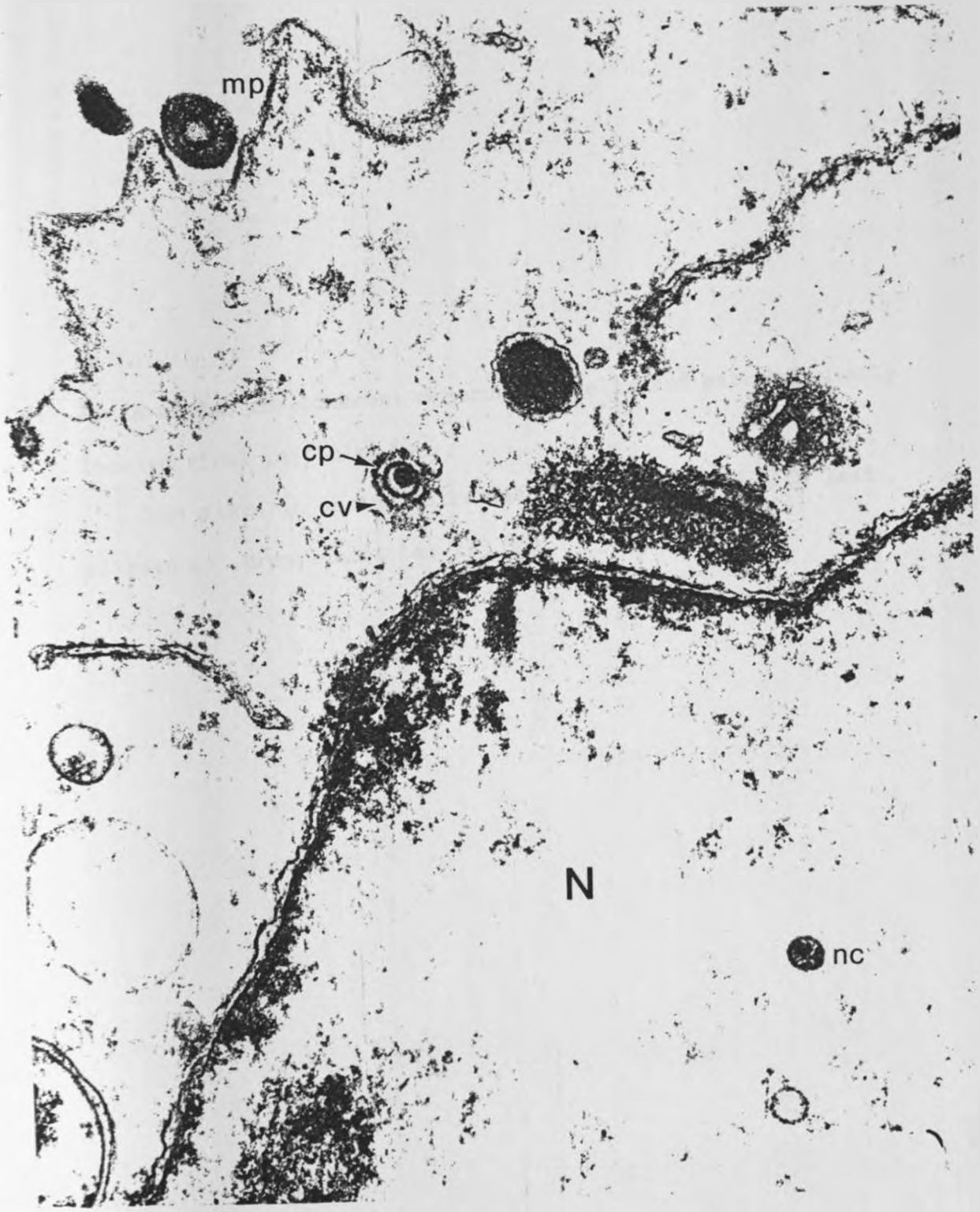

mp
cp
cv
N
nc

Fig. 26: Parts of two non-adherent mononuclear cells and extracellularly located viral particles (mp).

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x73,000.

N
M
M
mp
mp
mp

Fig. 27: Polymorphonuclear (PMN) cells culture in suspension and harvested 18 hours after infection with BHV-1. Some of the cells show moderate electronlucent central nucleoplasm whereas others do not.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x12,000.

cg = cytoplasmic granule.

cv = cytoplasmic vacuole

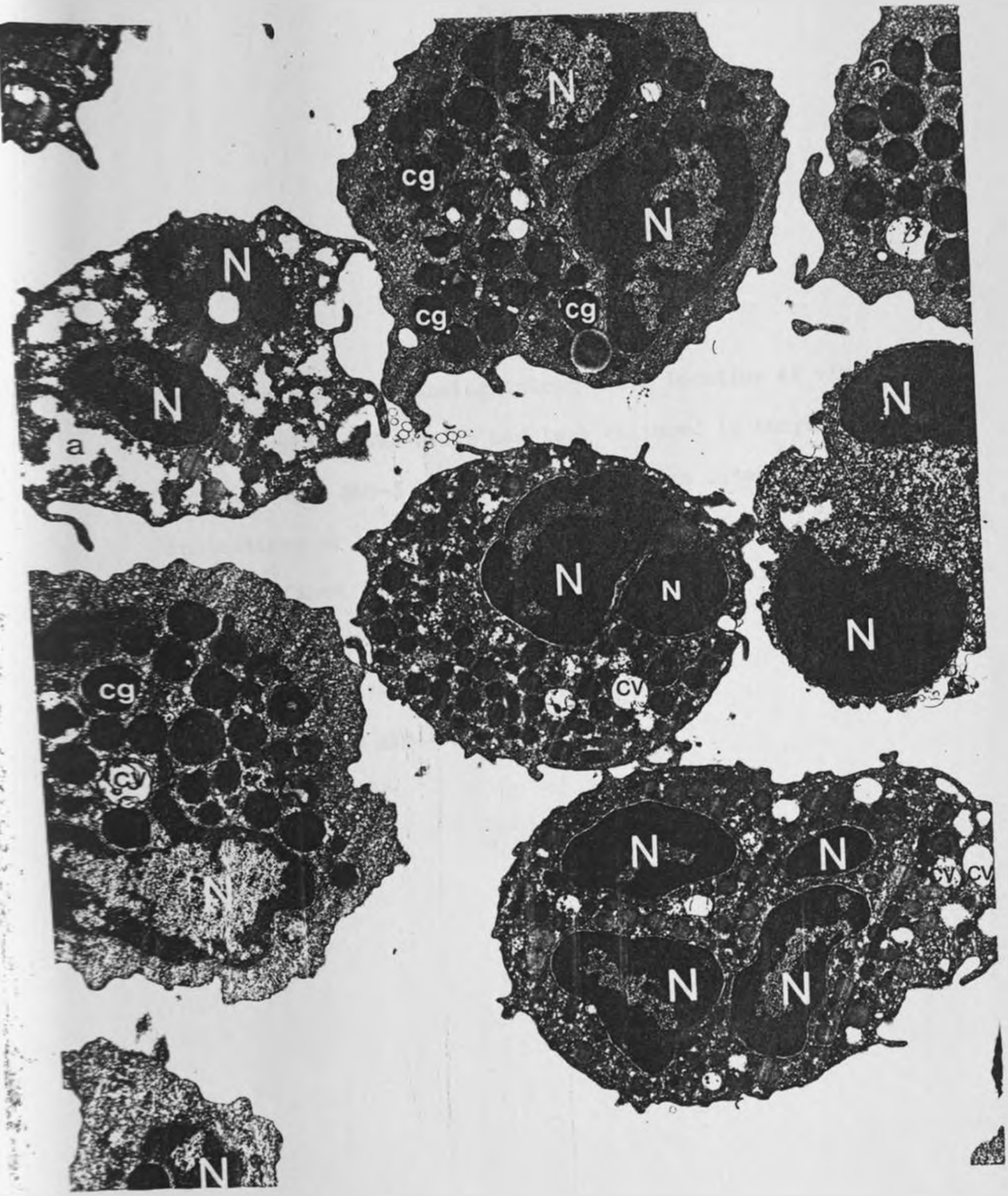

N
cg
N
N
cg
cg
N
N
a
N
N
N
cg
cv
N
cv
N
N
N
N
cv
cv
N
N
N

Fig. 28: Part of a PMN cell showing intranuclear location of viral nucleocapsids. The cells had been cultured in suspension, infected with BHV-1 and harvested 48 hours later. There are no indications of viral release.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x12,000.

mvc = multivesicular cytoplasm

sg = stain grains


N
M
sg
M
M

Fig. 29: Higher magnification of fig. 28. Notice the intranuclear location of viral nucleocapsids.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x77,000.

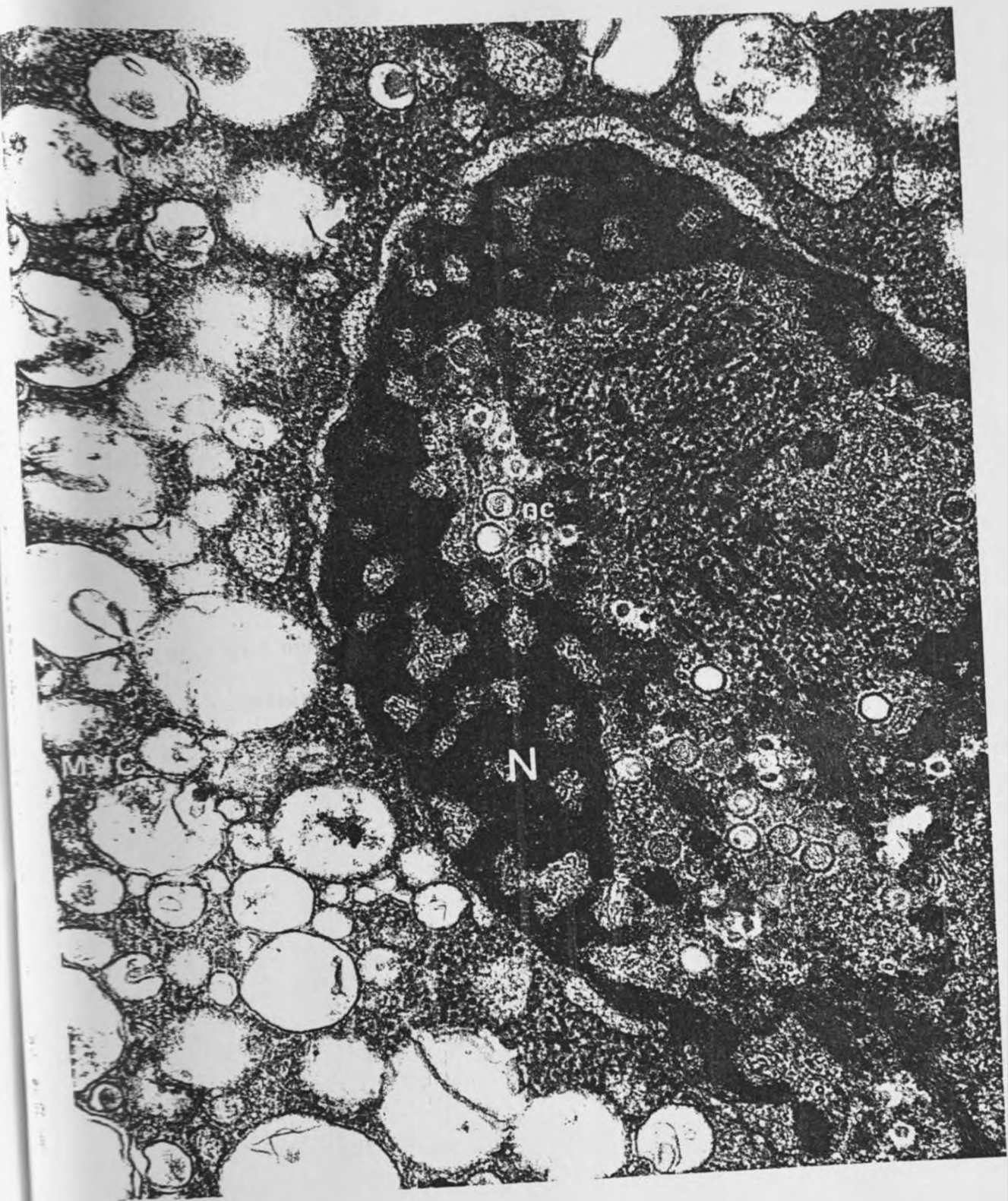

GC
N
MYC

Fig. 30: PMN cells co-cultivated with BEL cells which had been previously infected with BHV-1. The cells were harvested 9 hours after overlay on the BEL cells. Notice the large phagocytic vacuoles containing cell debris.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x19,000.

d = debris within vacuole

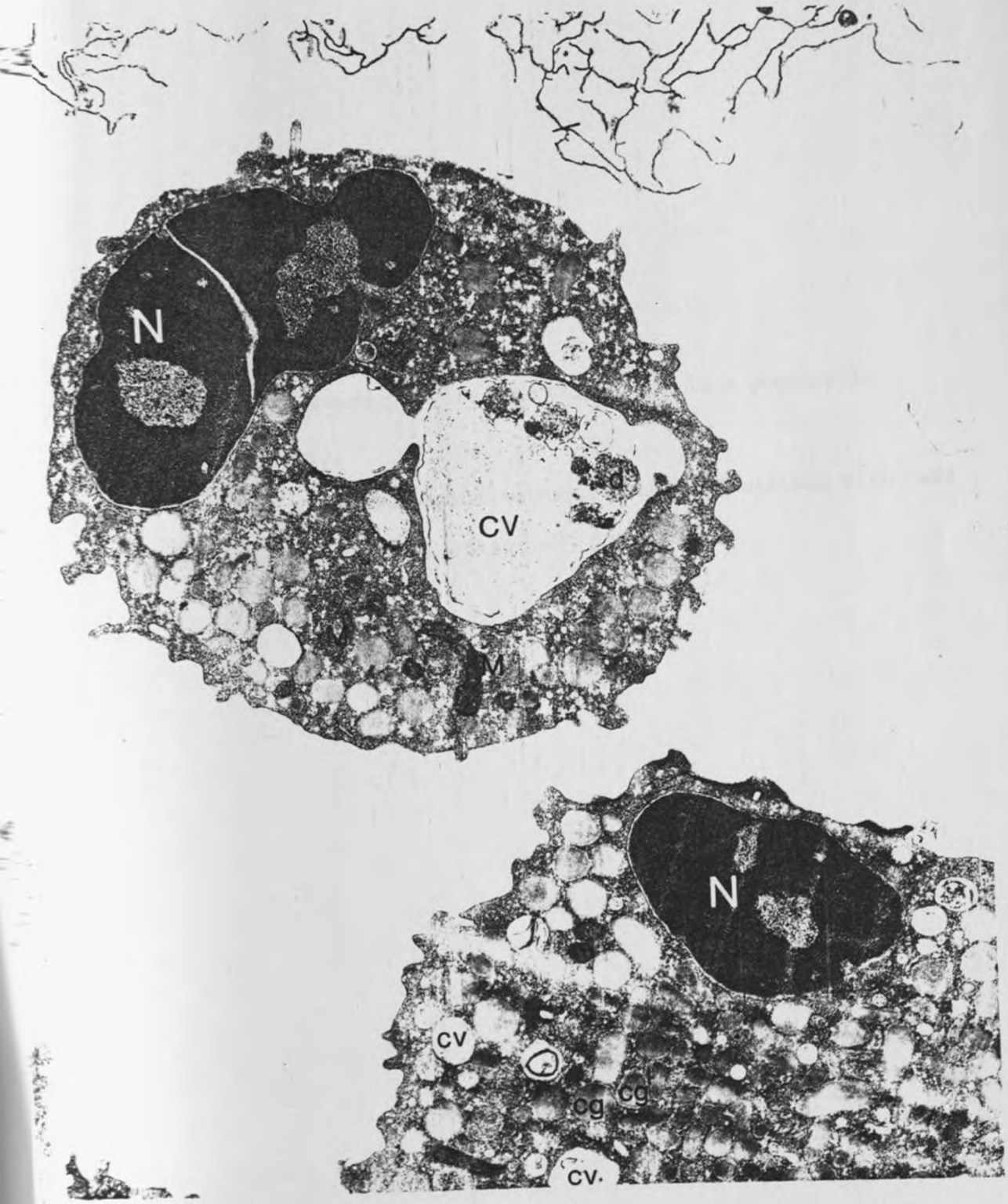

N
CV
M
N
cv
cg
cg
cv.

Fig. 31: PMN cells showing a viral particle (vp) in a phagocytic vacuole (cv).

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x73,000.

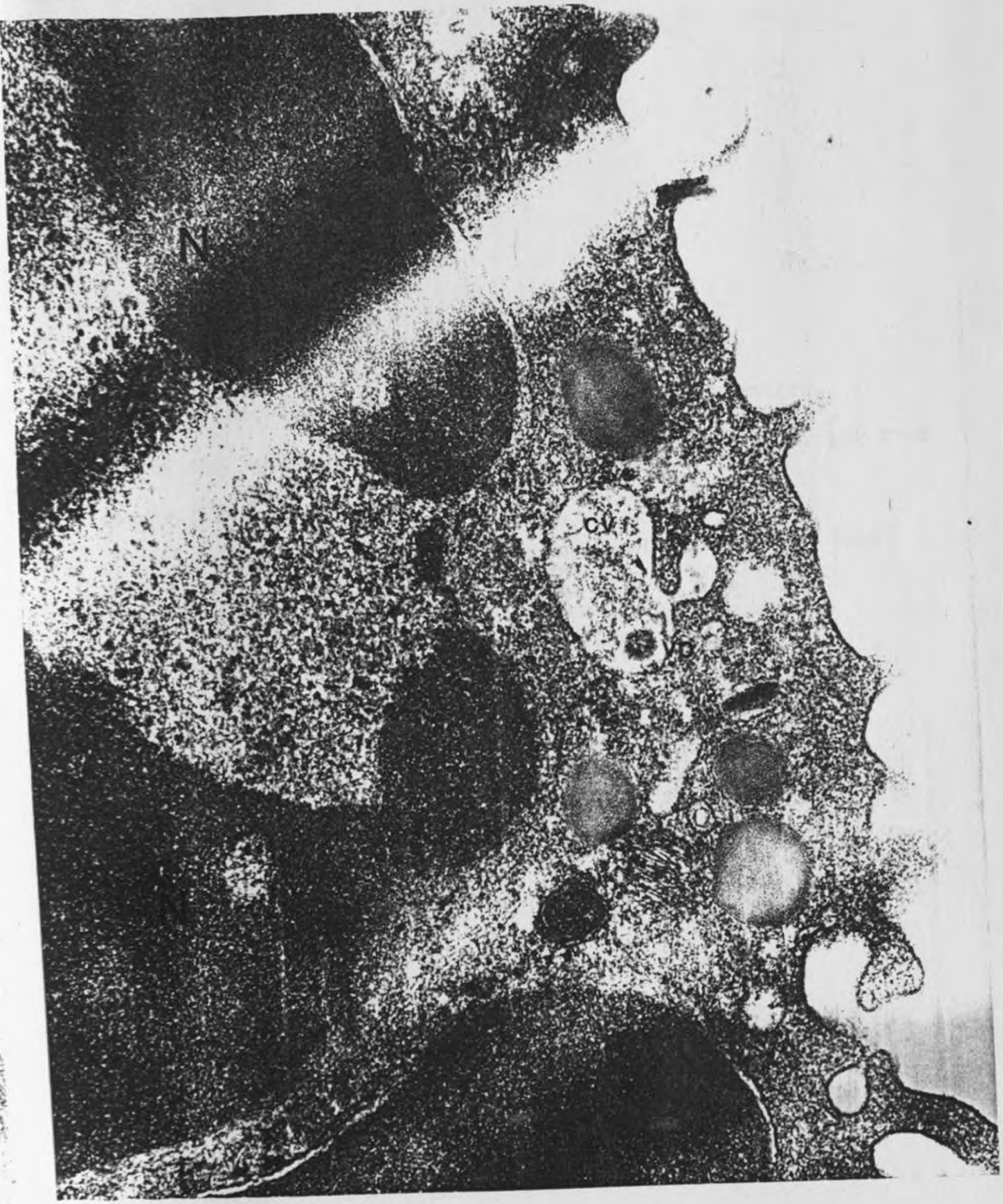
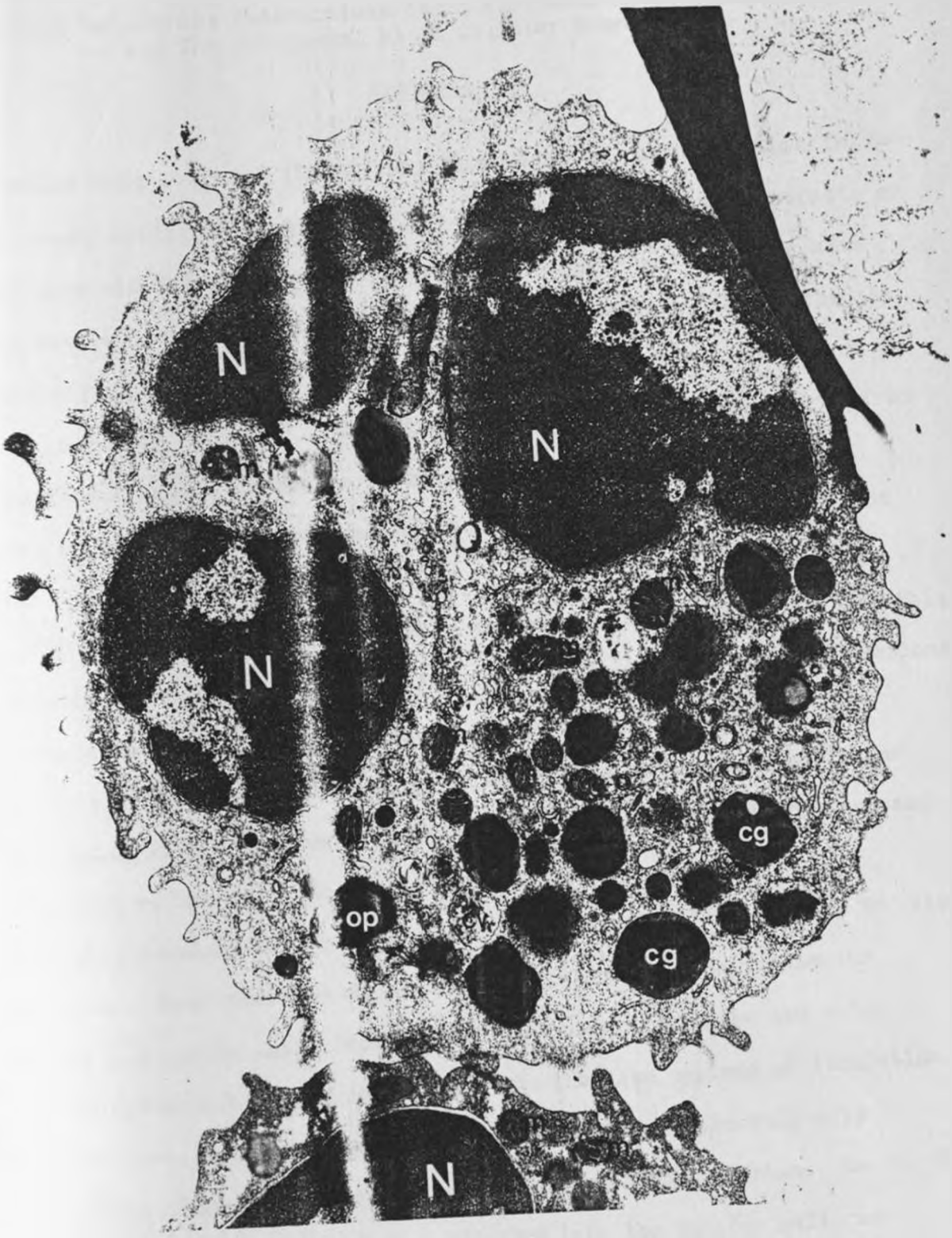

N
CV

Fig. 32: PMN cell, showing fine structure of cytoplasmic granules. Notice the "onion peel" (op) whorl-like structures resulting from poorly fixed granules.

Post fixed in osmium tetroxide and post stained with lead citrate and uranyl acetate; x25,000.


N
N
N
N
cg
cg
op

Philip Njeru Nyaga
December 1975
Comparative Pathology

In vitro Cell-virus Interactions Associated with Bovine Herpesvirus-1 and The Peripheral Blood Cellular Components

Abstract

Bovine herpesvirus-1 (BHV-1) has been shown to cause abortion following an acute attack of viral infection of the upper respiratory tract, or vaccination via the nasal or the intramuscular routes. The virus also causes balanoposthitis, enteritis, keratoconjunctivitis, rhinotracheitis and vaginitis. The virus is believed to reach the placenta and the brain via hematogenous transport. The virus has previously been recovered from the buffy coat cells of bovine blood. The manner in which the virus relates to these and other blood cells while in transit in blood is not known.

The purpose of the present study was to investigate the nature of this relationship. An *in vitro* correlate of the *in vivo* cell-virus interactions was simulated by isolating peripheral blood cells on a Ficoll-Hypaque gradient and testing their capacity to adsorb and act as host cells for BHV-1 replication. This was done after demonstrating both adsorption and replication of the virus in whole blood.

Unfractionated leukocytes and red cells were initially tested for viral adsorption. More than 60% of maximum adsorption was observed in the leukocytic fraction while less than 10% was observed in the red cell component after 6 hours of incubation. In the late periods of incubation, a slight increase in adsorption was observed in the leukocytic cell preparations while a decrease was noted in the red cell series. Later, the leukocytic fraction was further subdivided into the various cell components, namely polymorphonuclear (PMN) cells on the one hand, and the mononuclear (MN) cells on the other. The latter were further divided into

monocytes and lymphocytes and shown to readily absorb BHV-1. PMN cells were tested for viral adsorption and uptake indirectly by examining for viral particles in electron micrographs.

Evidence from electron microscopy showed that viral replication accompanied by release of infectious virus occurred only in monocytes and Phytohemagglutinin (PHA) stimulated (PS) lymphocytes. However, unenveloped viral nucleocapsids were also observed in the nuclei of non PS lymphocytes and occasionally in PMN cells. No viral particles were observed in PMN cells infected in suspension cultures prior to 48 hours of incubation. However, viral particles and cellular debris were seen in phagocytic vacuoles of PMN cells co-cultivated with bovine embryonic lung (BEL) cells. The latter had been infected at a high input multiplicity.

In growth curve studies, lymphocytes and PMN cells were maintained in stationary suspension cultures, while monocytes and BEL cells were grown on glass. Peak viral titers were observed after 12, 18 and 36 hours of incubation for monocytes, BEL cells and PS lymphocytes, respectively. However, no viral replication was observed in the PMN cell components.

It was concluded that BHV-1 is transported *in vivo* replicating in monocytes and absorbed to lymphocytes. The red cells and the PMN cells were considered to play no significant role in hematogenous transport of the virus. This would follow from the observations that neither did the red cells adsorb significant quantities of the virus, nor did they support viral replication; and that any virus attached to the PMN cells would be eventually internalised and probably destroyed.

Based on the results of the study and the pathobiology of the inflammatory response, a hypothesis was advanced to explain viral entry into the blood and its exit in order to reach the target organs. The hypothesis states the following:

Macrophages and lymphocytes leaving the infected respiratory passages carry BHV-1 replicating in the former and adsorbed to the latter cell types. The cells lodge in the local lymph nodes where further viral replication occurs. Monocytes, leaving via efferent blood vessels, and lymphocytes leaving through the efferent lymphatics of such lymph nodes eventually transfer the virus to the blood. Migrating mononuclear cells act in concert to transport the virus to the placenta and the brain. In the placenta, viral replication occurs in the highly sensitive fetal tissues. This subsequently leads to a fatal viral infection of the fetus. The dead fetus is soon aborted. In the brain local viral replication soon leads to encephalitis.

X. APPENDIX

STATISTICAL ANALYSES

A. COMPUTATION OF 50% END POINTS

The strength of a virus culture is judged by the extent to which it could be diluted before inoculated test cell cultures failed to indicate effects of viral growth. In such a test, serial dilutions of the culture are prepared and fixed volumes of these are inoculated into different groups of cultures. An endpoint is estimated from the proportion of cultures that become positive at each dilution.

In order to use the method of Spearman-Kaerber it is assumed that:

1. Variation exists among inoculated cultures
2. For any culture there is a level of virus dose above which infection occurs and below which no infection will occur (sometimes called the threshold);
3. Successive doses are fairly close together.

Let the dose level [negative logarithms (-logs)] be denoted by $x_1, x_2, \ldots, x_k$, where x_k contains most virus. Suppose n_i cultures are inoculated at dilution x_i and r_i of them show cytopathogenic effects (CPE). The proportion of positive responders is

$$p_i = \frac{r_i}{n_i} .$$ Then the log median tissue culture infectious dose ($TCID_{50}$), M, is estimated by

$$M = \Sigma \{(p_{i+1} - p_i)\left(\frac{x_i + x_{i+1}}{2}\right)\} \qquad (1)$$

If doses are equally spaced so that $x_{i+1} - x_i = d$ for all i, the estimating equation may be written

$$m = x_k + \frac{1}{2}d - d(\Sigma p_i). \qquad (2)$$

If the number of cell cultures per dose is also constant and equal to n, the estimating equation may be written

$$m = x_k + \frac{1}{2}d - \frac{d(\Sigma r_i)}{n} \quad . \tag{3}$$

$$= x_k - d\left(\frac{\Sigma r_i}{n} - \frac{1}{2}\right) \quad . \tag{4}$$

Equation (4) was used for computation of 50% endpoints for all viral assays. It is apparent that only the summation of positive responders (Σr_i) is necessary in this case. For example, if a tenfold dilution was followed by two fold series so that $x_k = -1.0$, $d = -0.3$, $n = 5$ and the following observations for responders were made:

5/5, 5/5, 4/5, 2/5, 0/5, 0/5; then

$\Sigma r_i = 16$. Substituting for the various values in equation 4 gives the following:

$$m = -1.0 - 0.3(16/5 - \tfrac{1}{2}) = -1.81$$

whose antilog provides the endpoint of 1:64.

The viral titers were expressed as $TCID_{50}$ per unit volume, (0.025 ml). In order to facilitate uniform comparisons with work in other laboratories, the titers were expressed as concentration per ml of undiluted culture. Normally, if the titer is $xTCID_{50}$ per y mls, the titer per ml is given by

$$x\ TCID_{50} \text{ per y ml} : \frac{1}{y} \tag{5}$$

which becomes

$$x\ TCID_{50} \text{ per } 0.025 \text{ ml} \cdot 1/0.025 \text{ or}$$

$$x\ TCID_{50} \text{ per } 0.025 \text{ ml} \times 40 \text{ for titers reported herein.}$$

Clearly, when the titers are expressed as log $TCID_{50}$ per 0.025, one only needs add 1.6 (log 40) to convert them to titers per ml.

B. THE GEOMETRIC MEAN

All virus assay determinations previously expressed as $TCID_{50}$ per unit volume of virus culture were subsequently transformed into a logarithmic scale. Geometric means $(\bar{x}_g)_j$ for the appropriate replicates were computed from the logarithmic transformations using the following relationship (82)

$$\log \bar{x}_{g_j} = \frac{\sum_{i=1}^{n_i} \log x_i}{n_j} , \tag{6}$$

where i = the number of replicates averaged

j = the rank of determinations within any sampling series;

this relationship essentially says that the logarithm of the geometric mean is the arithmetic mean of the logarithms of the individual observations.

The geometric mean $(\bar{x}_g)_j$ of a set of observations $(x_1, x_2, \ldots, x_n)_j$ is $(\sqrt[n]{x_1 \cdot x_2 \ldots x_n})_j$. If we let the quantity $(x_1 \cdot x_2 \ldots, x_n)$ be represented by Q, then the geometric mean

$$\sqrt[n_j]{Q_j} = Q_j^{\frac{1}{n_j}} \tag{7}$$

Thus,

$$\log \bar{x}_{g_j} = \log Q_j^{\frac{1}{n_j}}$$

$$= \frac{1}{n_j} \log Q_j \tag{8}$$

Equation (8) is the easiest to use if tables for the appropriate nth roots are unavailable.

If we substitute for $\log \bar{x}_{g_j}$ in equation (6) we obtain the following:

$$\frac{1}{n_j} \log Q_j = \frac{\sum_{i}^{n_j} \log x_i}{n_j} \tag{9}$$

The antilog of the term on either side of equation (9)provides the

C. EVALUATION OF SIGNIFICANCE

Since most determinations were made in pairs, Wilcoxon's signed rank test for paired data was used to test for significance. The n pairs of observations were assumed independent. The tested hypothesis is that the first and the second members of the pairs came from populations having the same median, or that the pair differences come from a population having a median zero, i.e.

$$H_0: Md_1 = Md_2$$

$$H_A: Md_1 \neq Md_2 ,$$

or $H_0: Md_1 - Md_2 = 0$

$H_A: Md_1 - Md_2 \neq 0$.

Any ties in paired entries were removed from the data before analysis. Thus, n became the number of non-tied pairs. T_+, the sum of the positive ranks is then the test statistic.

The computations are shown in Tables A.1 to A.3 and the conclusions are summarised below each table.

Table A. 1.0 VIRUS TITER* FOLLOWING INOCULATION OF BHV-1 INTO BOVINE BLOOD AND PLASMA

	Sampling Time											
	3	6	9	12	15	18	21	24	27	30	33	36
Blood	3.0	3.8	5.7	8.6	12.8	10.2	8.1	12.8	11.6	14.9	20.3	25.6
Plasma	6.4	8.1	6.4	5.1	6.4	6.4	4.0	4.0	3.2	2.0	1.6	1.0
Difference	3.4	4.3	0.7	3.5	6.4	3.8	4.1	8.8	8.4	12.9	18.9	24.6
Sign of Difference	-	-	-	+	+	+	+	+	+	+	+	+
Rank	2.0	6.0	1.0	3.0	7.0	4.0	5.0	9.0	8.0	10.0	11.0	12.0

$T_+ = 69$.

Critical values for $n = 12$, $T_{+0.05} = 13$ and 65; $T_{+0.02} = 9$ and 69 ,

$\therefore \quad 0.02 \leq P < 0.05$.

*: In log $TCID_{50}$ per ml

Table A. 2.0 VIRUS TITER* FOLLOWING INOCULATION OF BHV-1 INTO BEL CELLS AND CONTROL MEDIA

	Sampling Time													
	0	3	6	9	12	15	18	21	24	27	30	33	36	39
BEL Cells	4.4	4.8	4.5	7.1	7.4	7.8	7.8	7.7	7.2	7.1	7.5	7.7	7.6	7.2
Control	4.5	4.1	4.1	4.4	4.7	4.6	4.3	4.2	4.0	3.9	3.7	3.3	2.9	2.2
Difference	0.1	0.7	0.4	2.7	2.7	3.2	3.5	3.5	3.2	3.2	3.8	4.4	4.7	5.0
Sign of difference	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Rank	1	3	2	4.5	4.5	7	9.5	9.5	7	7	11	12	13	14

$T_+ = 104$

Critical values, n = 14, are 21 and 84; 15 and 90; and 12 and 90 for $T_{+0.05,0.02,0.01}$ respectively.

$\therefore P \leq 0.01$.

*: In log $TCID_{50}$ per flask

Table A. 3.0 VIRUS TITER* FOLLOWING INOCULATION OF BHV-1 INTO BEL AND AD-MN CELLS

	Sampling Time									
	0	3	6	9	12	15	18	21	24	27
AD-MN Cells	3.9	4.5	4.8	5.0	5.2	5.1	4.9	4.7	4.4	4.1
BEL Cells	4.4	4.8	4.5	7.1	7.4	7.8	7.7	7.8	7.2	7.1
Difference	0.5	0.3	0.3	2.1	2.2	2.7	2.8	3.1	2.8	3.0
Sign of Difference	-	-	+	-	-	-	-	-	-	-
Rank	3.0	1.5	1.5	4.0	5.0	6.0	7.5	10.0	7.5	9.0

$T_+ = 1.0$

For $n < 10$, critical values for $T_{+0.05,\ 0.02,\ 0.01}$ indicate that

$P \leq 0.01$.

*: In log $TCID_{50}$ per flask.