AN ABSTRACT OF THE THESIS OF

John E. Ward Jr. for the Master of Science

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Title: The Interaction of Stimulated Mouse Macrophages with Vaccinia Virus In Vitro

Abstract approved: Helen McCluskey PhD

Stimulated mouse peritoneal macrophages were studied to determine the mechanism by which they express resistance to vaccinia virus. Macrophages from non-immunized animals were harvested and infected with vaccinia *in vitro*. The virus failed to replicate within the macrophage culture. Autoradiographic studies revealed that no significant viral DNA synthesis occurred in infected macrophages. An uncoating assay yielded evidence that vaccinia did not undergo replicative second stage uncoating within the mouse macrophage. Examination of infected macrophages with the electron microscope indicated that vaccinia was retained within the phagocytic vacuole of the macrophage where it underwent at least partial degradation. Because no portion of the viral replicative sequence was detected, the stimulated mouse macrophage appears to be a resistant rather than a non-permissive

cell type for vaccinia. It is suggested that the level of mouse macrophage activation may be an important factor in vaccinia resistance.

THE INTERACTION OF STIMULATED MOUSE MACROPHAGES WITH VACCINIA VIRUS IN VITRO

A Thesis

Submitted to

the Division of Biology

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by

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ACKNOWLEDGEMENT

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INTRODUCTION

Host resistance to invasion by a pathogen comprises a complex interaction of several protective mechanisms. One of the most basic, but important, of these mechanisms is that which operates at the cellular level through the process of phagocytosis.

The phagocytic theory was first proposed by Metchnikoff in 1884 (Hirsch, 1959) as a result of his observation that Daphnia could ingest and destroy certain pathogenic yeasts. Metchnikoff's work established the macrophage as a primary phagocytic cell type. This finding was expanded by van Furth (1970) to include a heterogeneous collection of cells which he termed the mononuclear phagocytes. These cells include circulating peripheral monocytes, precursor cells in the bone marrow, and tissue macrophages, all of which are characterized by a specialized ability to ingest and dispose of foreign material. In this sense foreign material may include effete host cells or host cell debris along with foreign microorganisms.

The importance of the macrophage in defense of the host against viral infections has become increasingly clear within the last three decades. Dalldorf (1950) showed that newborn mice were highly susceptible to coxsackie virus although adult mice were resistant to infection. It was suggested that this difference was due to the development of a functional

macrophage system in the mature animals. Similar results were reported by Johnson (1964), who did an *in vitro* study of the susceptibility of peritoneal macrophages from suckling and adult mice to Herpes virus. Fluorescent antibody techniques demonstrated that infection occurred less frequently and developed slower in cells as the animals' age increased. A parallel study using autoradiography revealed that no radioactive label was present in infected adult macrophages while suckling macrophages contained wide-spread labeling, indicating viral DNA production in these cells. No substance in adult mouse serum was found to interfere with infection and Johnson concluded that the resistance which developed was a function of the age of the macrophage donor and, consequently, maturation.

Bang and Warwick (1960) also demonstrated the importance of macrophages in dealing with viral infections. Liver macrophages from a susceptible mouse strain were found to degenerate when infected *in vitro* with mouse hepatitis virus. Macrophages from resistant mice were found to survive infection, suggesting a genetic difference in susceptibility of the two mouse strains which probably operated at the macrophage level. This theory was reinforced by the work of Theis and Koprowski (1961) using macrophages from both susceptible and resistant mice strains to West Nile virus. Little virus was produced by splenic and peritoneal macrophages from resistant mice although large amounts of virus was obtined from macrophages of susceptible mice. Further

evidence of the role of macrophages was presented by Roberts (1963) from an antibody study of the differences between the avirulent hampstead egg and the virulent hampstead mouse strain of ectromelia virus. His work revealed that the inability of the avirulent virus strain to initiate infection was due to the resistance of mouse liver macrophages to the virus.

Vaccinia, because it is a well characterized and relatively large virus, has been used extensively in studies concerning macrophage-virus interactions. Nishmi and Bernkopf (1958) reported that mouse splenic macrophages exhibited resistance to vaccinia infection. This finding was confirmed by Nishmi and Niesikowski (1963) who demonstrated that normal mouse peritoneal macrophages would not support vaccinia replication. This work indicated a species specific difference in macrophage resistance to vaccinia since earlier studies by Beard and Rous (1938) had shown that normal rabbit peritoneal macrophages allowed replication of vaccinia in vitro.

Further insight into this difference in macrophage resistance was obtained when Steinberg and Rights (1963) reported that spleen cell cultures from rabbits immunized against vaccinia were more resistant to infection than were similar cultures from normal rabbits. Humoral factors were shown not to be a factor in this resistance. Because the spleen contains large numbers of macrophages, these results suggested that macrophages from immunized rabbits had acquired

a cellular immunity to vaccinia. Later work by Tompkins *et al*. (1970) and Avila *et al*. (1972) supported this theory by demonstrating that peritoneal macrophages from vaccinia-immune rabbits were resistant to infection by vaccinia but not myxoma virus. This indicated that the development of specific macrophage resistance had occurred. This resistance has since been attributed to macrophage activation resulting from a lymphocyte-mediated immune response.

However, as previously described, mouse peritoneal macrophages displayed a natural resistance to vaccinia which was not dependent upon an immune response. This implied that the protective mechanism employed by mouse macrophages was different from that which occurred in the immune rabbit macrophage. Although these findings represent a basic disparity amoung forms of cellular resistance to viral challenge, only a limited amount of work has been done toward understanding the manner in which the mouse macrophage inhibits vaccinia replication. Some information, however, has been obtained.

Schultz (1966) investigated the fate of vaccinia within infected mouse macrophages *in vitro*. He found that virus was phagocytized by macrophages in which it displayed a continuous decrease in infectious titer until by 116 hr no virus could be recovered within the system. Flourescent antibody studies of infected cells revealed a similar pattern with the disappearance of labeled viral antigens by 70 hr post-infection. Schultz then compared the activity of

lysosomal enzymes within both infected mouse and rabbit macrophages using acid phosphatase stain and found that the rabbit cells displayed a greater incidence of lysosomal activation than did mouse cells. From these results he postulated that the difference in macrophage susceptibility was due to the presence of different kinds and amounts of lysosomal enzymes in the two cell types. This resulted in viral degradation in mouse macrophages but allowed viral replication to proceed in rabbit macrophages.

However, on the basis of his observations using the electron microscope, Silverstein(1970) reported that vaccinia underwent first stage uncoating within the mouse macrophage as evidenced by the appearance of viral cores within the cell cytoplasm. Parallel biochemical experiments using purified ^{3}H-thymidine labeled virus showed that uncoating of viral DNA had not occurred, although thymidine kinase activity was induced within the macrophage 1 hr following infection. Macrophage lysis occurred 3 to 4 hr after infection, depending upon the multiplicity employed. Silverstein's work indicated that vaccinia was not degraded by lysosomal enzymes but was inhibited during the replicative sequence between first and second stage uncoating. Macrophage death then occurred from a toxic effect caused by the build up of viral antigens.

A still different scenario was proposed by Mackaness and Raffel (1971) when they reported that vaccinia was phagocytized and quickly inactivated within mouse macrophages. Although the virion was partially uncoated within the

phagosome, the viral core was not released into the cell cytoplasm. Cell death then occurred from a viral-induced toxic effect. These results indicated a possible role of cell lysosomal enzymes both in inactivating the virus and in causing the subsequent breakdown of the phagosome membrane.

Due to the important role of the macrophage in host defense against viral disease, it is vital to understand the mechanism by which this cell type resists infection. Macrophages have additional functions; indeed, they appear to be associated with many immunological processes including delayed hypersensitivity, induction and implementation of primary antibody responses, and cell-mediated cytotoxicity. However, it was the phagocytic and antimicrobial activity which was of primary interest in the present study. Because of the conflicting results obtained concerning the fate of vaccinia within the mouse macrophage, it was of interest to further investigate this host-pathogen interaction.

MATERIALS AND METHODS

Experimental Animals

Young adult white mice of both sexes were obtained from the Emporia State University animal room. Six to eight month old New Zealand white rabbits were obtained from breeders in the Emporia area.

Cell Culture Medium

All cell types used in this study were cultured in Eagles Minimal Essential Medium (MEM) containing L-glutamine and sterile fetal calf serum at a final concentration of 10 percent. Hanks balanced salt solution (HBSS) plus two units of heparin per ml was used for the harvesting of both mouse and rabbit macrophages. For the preparation of mouse embryo fibroblasts Dulbeccos' phosphate buffered saline (DPBS) without calcium or magnesium ions was employed. All culture solutions contained Combiotic (penicillin, 200 units per ml; and streptomycin, 100 units per ml). When needed, solutions were adjusted to pH 7 with either 1.5% or 7.5% sterile sodium bicarbonate.

Cell Culture Procedures

(1) Mouse Fibroblast Culture

Pregnant mice were killed by cervical dislocation. The abdominal hair and skin were saturated with NIH disinfectant and the ventral skin laid back. The abdomen was

opened to expose the uteri and the embryos were removed and placed in a sterile petri dish containing DPBS. The embryos were decapitated, eviscerated, and transferred to another petri dish containing fresh DPBS. After mincing with sterile scissors, the embryo fragments were placed in a trypsinizing solution consisting of DPBS plus 0.25% trypsin and 0.02% EDTA. The tissue fragments were trypsinized with stirring for 15 minutes. The resulting cell suspension was filtered through sterile cheesecloth into a flask containing DPBS plus 10% serum to halt the action of trypsin. More trypsinizing solution was added to the remaining fragments and the procedure repeated until digestion was complete. The cell harvest was washed twice by centrifuging at 1500 rpm for 10 min and resuspended in DPBS. After adjusting to 2 X 10^6 cells per ml with Eagles' MEM the cell suspension was planted in Roux bottles at 50 ml per bottle. The cells were incubated at 37°C until monolayered, usually 39 to 48 hours.

(2) Mouse Peritoneal Macrophage Culture

Experimental mice were given an intraperitoneal injection with 2 ml of 3% sterile thioglycolate 4 days prior to harvest (Fig. 1). Animals were sacrificed by cervical dislocation and secured to a work board. The abdominal hair was saturated with NIH, and the ventral skin laid back (Fig. 2). The exposed muscle layer was swabbed with NIH and 4 ml of heparinized HBSS were injected into the peritoneal cavity (Fig. 3). The abdomen was massaged with NIH soaked cotton to suspend peritoneal cells and a small incision made in

Figure 1: Intraperitoneal injection of thioglycolate solution.

Figure 2: Experimental mouse tacked to operating board for the harvest of peritoneal macrophages.

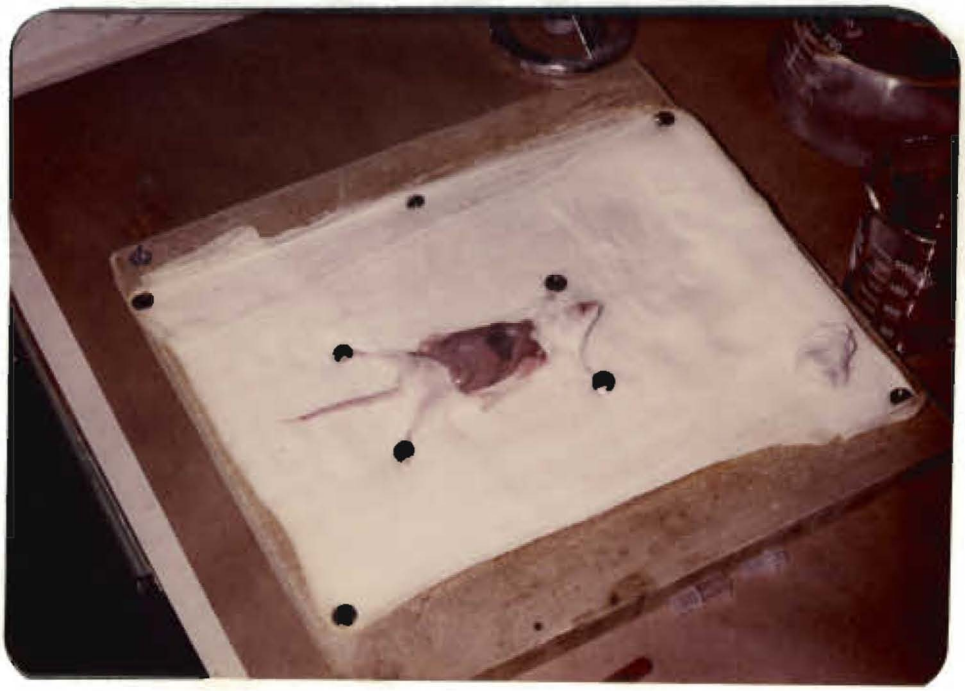


Figure 3: Injection of HBSS into the peritoneal cavity for macrophage harvest.

the peritoneal wall along the midline. The cell-containing fluid was collected using capillary pipettes (Fig. 4), and the harvest from all animals pooled. Mononuclear cells were counted and the number adjusted to 3×10^6 cells per ml with HBSS. Cells were planted in either flying coverslip tubes or plastic cell culture flasks, in 1 ml or 5 ml amounts respectively, and allowed to settle for 1 hr at 37°C. The adherent cells were then washed twice with HBSS without heparin and the medium replaced with Eagles' MEM.

(3) Rabbit Peritoneal Macrophage Culture

Experimental rabbits were injected intraperitoneally with 50 ml of sterile mineral oil (Squibb Heavy Duty) 5 days prior to harvest. The animals were sacrificed by embolism produced through the injection of air into the marginal ear vein. The abdominal hair and skin was saturated with NIH and the hair removed. The abdominal skin was laid back and the underlying tissue swabbed with disinfectant. Fifty ml of heparinized HBSS were injected into the peritoneal cavity and the abdomen was massaged to dislodge peritoneal macrophages. The fluid was removed with a sterile 50 ml volumetric pipette through an incision in the peritoneal wall and placed in a separatiory funnel. The funnel was placed at 4°C for 10 min to allow the oil to separate from the cell layer. The cell suspension was then drained into 50 ml polycarbonate centrifuge tubes and centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted and the cells washed with HBSS and recentrifuged. The cells were then

Figure 4: Removal of cell suspension from the peritoneal cavity.

resuspended in HBSS, pooled, and counted. The cell suspension was adjusted to 3 X 10^6 cells per ml with HBSS and planted in tissue culture flasks at 5 ml per flask. After settling for 1 hr at 37°C, the cells were washed twice with HBSS and the supernate replaced with Eagles' MEM.

Virus

(1) Propagation

The IHD strain of vaccinia virus used in this study was originally obtained from the American Type Culture Collection. Mouse embryo monolayers were infected with 1 X 10^4 PFU of virus in 10 ml of Eagles' MEM plus 2% serum. The inoculum was allowed to remain in contact with the cells for 2 hr with frequent agitation of the bottles to promote viral absorption. The infecting medium, along with any unabsorbed virus, was removed and replaced with Eagles' MEM containing 10% serum. The monolayers were incubated at 37°C until extensive cellular destruction was observed (24 to 36 hr).

Radioactively labeled virus was prepared by harvesting and infecting mouse embryo monolayers. After viral absorption and cell washing, 50 ml of Eagles' MEM containing ^{3}H-thymidine (New England Nuclear) at 200 µc per ml was added to each monolayer. The cells were then incubated at 37°C until viral-induced cell destruction was complete.

(2) Harvesting of Virus

When the destruction of the infected cell monolayers was complete the Roux bottles were frozen, cell side down,

at -70°C. The bottles were then thawed at room temperature with frequent agitation to remove the cells from the glass. The virus-cell suspension was sonified for 1 min at 0.9 amperes in a Raytheon Sonic Oscillator (Model DF-101) to release cell associated virus, followed by centrifugation at 3500 rpm for 10 min to remove cell debris. The supernates were pooled, aliquoted in appropriate amounts, and stored at -70°C until use.

(3) Assay of Virus

Mouse embryo fibroblasts were harvested and monolayered in Corning plastic culture flasks at 2 X 10^6 cells per ml. The culture medium was removed and the cells washed twice with HBSS, Appropriate dilutions of the virus to be titered were prepared in Eagles' MEM containing 2% serum and 1 ml amounts were placed on monolayer cells. Viral absorption was allowed to occur for 2 hr with frequent agitation of the flasks. Four ml of Eagles' MEM with 10% serum were then added to each flask. The monolayers were incubated at 37°C for 36 to 48 hr until plaque formation was obvious. The medium was removed and the cell layer stained with 2 ml of a 1:10 aqueous crystal violet solution. The flasks were rinsed with tap water, dried, and the plaques present on countable dilutions counted. Viral titer was calculated and expressed as plaque forming units (PFU) per ml.

Infection of Macrophages

Following the attachment of macrophages, cellular debris

and non-adherent cells were removed by two washes with HBSS. The virus inoculum was diluted in Eagles' MEM containing 2% serum to the desired multiplicity and added to the macrophage cultures at 1 ml per coverslip tube and 5 ml per culture flask. After a 2 hr absorption period at 37°C, the infecting medium was decanted and the cells washed twice with HBSS to remove any unabsorbed virus. The medium was replaced with Eagles' MEM and the cells returned to 37°C. A sample of viral inoculum was retained in order to establish zero hr concentration. Zero hr was taken to be the time virus was added to the cells.

Coverslips were pulled periodically for cell staining and for determination of growth curves. Coverslips were pulled at 2 hr after infection, when complete medium was added, to determine the original amount of cell-associated virus in the system. Thereafter, coverslips were sampled at 24 hr intervals. Virus from each time period was titrated by three cycles of freeze-thaw and assayed on mouse embryo monolayers.

Macrophage coverslips to be used in autoradiography were allowed to settle and infected at a multiplicity of infection (MOI) of 1.0 by the procedure previously described. After the 2 hr absorption period, the infecting medium was removed and replaced with Eagles' MEM containing 1 μc per ml of tritiated thymidine.

Macrophage cell culture flasks to be used in the second stage uncoating assay were allowed to settle for 2 hr at 4°C,

washed twice with cold HBSS, and infected with 1 ml of ^{3}H-thymidine labeled vaccinia (1.28×10^{-2} CPM per PFU) in cold Eagles' MEM containing 2% serum at a MOI of one. Following viral absorption for 2 hr at 4°C, residual virus was removed by two washes with cold HBSS and the medium replaced with warm (37°C) Eagles' MEM containing 10% serum. The flasks were then incubated at 37°C to synchronize viral phagocytosis.

Mouse macrophage culture flasks to be used in electron microscopy were infected after settling at a MOI of one hundred. Rabbit macrophages used in electron microscopy were infected at a MOI of fifty. Both cell types were then handled as previously described.

Staining of Cells

Coverslips were air dried, fixed in absolute methanol (5 min), stained with May Grünwald (9 min) and Giemsa (14 min), dehydrated in acetone and in 50:50 acetone-xylene, cleared in xylene, and mounted cell side down on microscope slides with Permount mounting medium.

Autoradiography of Macrophages

Uninfected control and infected macrophage coverslips to be used in autoradiography were pulled at 8, 24, 48, and 72 hr and prepared by first washing them twice in cold HBSS and fixing for 15 min in cold 2.5% gluteraldehyde at 4°C. This was followed by two washes with cold HBSS. The cells were next placed in cold 2% perchloric acid for 30 min at 4°C and washed with three changes of distilled water for 10

minutes. After being air dried, the coverslips were mounted cell side up on microscope slides with Permount. Prior to mounting, the backs of the coverslips were gently cleaned with lens paper. The slides were dipped once into a chrom alum solution (5% gelatin, 0.5% chrom alum) and allowed to dry for 2 days in a horizontal position.

In the darkroom the slides were warmed to 45°C, coated with warm NTB-3 emulsion (Eastman Kodak Co.), and shaken twice to remove excess emulsion. The backs of the freshly dipped slides were wiped clean and the slides allowed to dry for 30 min under cool air flow from a hair dryer before being placed in light tight boxes and stored at 4°C for 10 days. After exposure, the slides were developed in the darkroom in D-19 (Eastman Kodak Co.) for 10 min, washed and fixed in Hypo Fixer (Eastman Kodak Co.) for an additional 10 min, and washed again for 15 minutes. The slides were air dried and stained with Giemsa for 15 min, washed for 30 sec, stained for 1.5 min with Delafields' Hematoxylin and washed for 1 min in saturated lithium carbonate. Dehydration was accomplished by placing the slides in successive washes of 70%, 95%, and 100% ethanol for 10 min each followed by clearing with xylene for an additional 10 minutes. The finished slides were then covered with coverslips using Permount and examined for clusters of silver grains in the cells. The amount of nuclear and cytoplasmic labeling was determined by finding the percent of macrophages containing labeling per 5000 cells counted.

Vaccinia Second Stage Uncoating Assay

To determine if viral DNA had been released from the viral core after phagocytosis, a second stage uncoating assay was performed. The method used was a modification of that developed by Joklik (1963) and consisted of an assay to determine whether viral DNA was present within the macrophage in a form susceptible to DNase enzyme. The test utilized the fact that trichloracetic acid (TCA) will precipitate large molecules. Labeled viral DNA, which was present in an intact form, is acid insoluble and could be precipitated onto filter paper. Hydrolyzed viral RNA and DNase degraded viral DNA, however, would be acid soluble and thus remain in the TCA solution. Thus, the amount of radioactive label found in the filter paper would represent the amount of protected viral DNA, or that DNA which had not been released from the core.

Duplicate infected flasks were processed at 4, 6, and 8 hr by first removing the cells from the flask surface with a rubber policeman, pooling the cell suspensions, and centrifuging at 2000 rpm for 10 minutes. The cell pellet was resuspended in one half volume of cold buffer (0.01 M $NaPO_4$; 0.01 M $MgCl_2$, pH7) and sonified at 0.9 amperes for 30 seconds. The resulting lysate was processed in two ways. A 1 ml sample was incubated with DNase (200 µg per ml) for 30 min in a 37°C water bath, and another 1 ml sample was incubated with buffer as a control. Both samples were then prepared for counting by the method of Sobieski and Olsen (1973). This

procedure involved first hydrolyzing viral RNA by incubating duplicate 0.1 ml amounts of the samples with 0.15 ml of 0.75 N KOH for 12 hr at 37°C. The samples were then absorbed into three cm^2 filter papers (Whatman #1) which were secured by pins pushed through cardboard. The papers were dried for 30 min at 60°C and placed face down, one at a time, into a large beaker containing cold 10% TCA (10 ml per filter paper) in an ice bath. The flask was swirled every 5 min for a total of 30 minutes. The papers were then transferred individually to another flask containing cold 5% TCA (5 ml per filter paper) for 20 min, again with frequent swirling of the flask. Sample papers were dehydrated by placing them in 50 ml of a 1:1 ethyl ether-100% ethanol mixture for 5 min and air dried. The papers were placed in scintillation vials containing 15 ml of cocktail (PPO-POPOP, Research Products International) and the amount of radioactive label present determined by counting in a Nuclear Chicago Unilex II Liquid Scintillation Counter.

Electron Microscope Techniques

Mouse macrophages were sampled at 2, 4, and 6 hr after infection while rabbit macrophages were sampled 3 hr after infection. The cells were processed by decanting the medium and washing twice with cold 0.1 M phosphate buffer (pH 7.4). Cold 4% phosphate buffered gluteraldehyde (pH 7.4) was then added and left on the cells for 1 hr at 4°C. The cells were next washed five times with cold buffer and post fixed for

30 min in cold 1% phosphate buffered osmium tetroxide at 4°C. After three washes with cold buffer 2 ml of buffer were added to each flask and the cells scraped loose with an applicator stick. The contents of several flasks were pooled in a conical centrifuge tube and centrifuged at 2000 rpm for 10 minutes. The supernate was carefully decanted and the cell pellet resuspended in the tube with two drops of warm 2% agar, then placed at 4°C to allow the agar to harden. The agar block was removed from the tube and cut into 1 mm^2 pieces. The cell clumps were then placed in cold 50% ethanol for 30 min followed by staining in a saturated solution of uranyl acetate in 70% ethanol for another 30 minutes. The cells were dehydrated further by placing them in changes of 95% and 100% ethanol, then two changes of cold propylene oxide, all at 4°C for 30 min each. The cell blocks were brought to room temperature and placed in a 50:50 mixture of Poly Bed 812 Embedding Media (Polysciences, Inc.) and propylene oxide within a loosely covered container to allow for the overnight evaporation of propylene oxide. Fresh complete Poly Bed 812 was placed on the cell blocks for 2 hr before placing them in Beem capsules and filling with fresh Poly Bed 812. Curing was accomplished at 60°C for 24 hours. The blocks were trimmed and sectioned on an IKB ultratome. The sections were collected on 200 mesh grids, allowed to air dry, and stained with Reynolds' lead citrate for 1 min followed by two washes with distilled water. The sections were examined and photographed with a Hitachi HS-8 Electron

Microscope. Kodak contrast 3¼" X 4" projector slide plates were exposed and developed in D-19. The negatives were then enlarged on Kodak F-5 Kodabromide paper.

RESULTS

Morphology of Macrophages

Stimulated mouse peritoneal macrophages, when stained after settling and washing, appeared as rounded, moderately vacuolated cells of varying size possessing a rather large nucleus of heterogeneous shape ranging from oval to highly indented (Fig. 5). During the course of *in vitro* cultivation cell density tended to decrease, there was an overall increase in cell size, and some cells exhibited pseudopod extensions, especially in less crowded areas of the coverslip. After 96 hr in culture approximately one half of the cell population exhibited this extended morphology (Fig. 6).

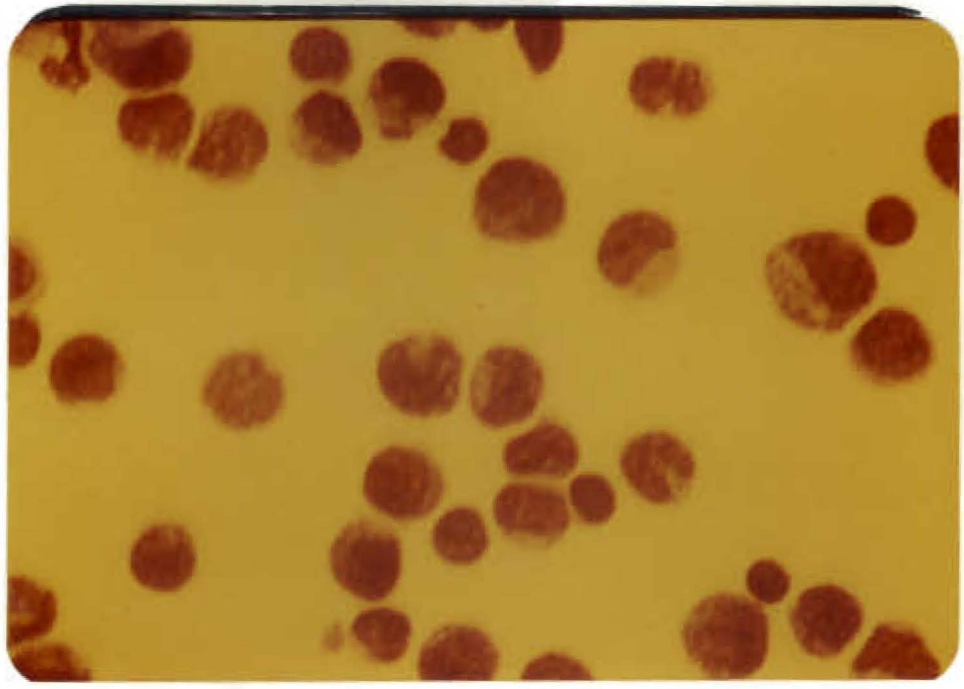
Macrophages infected with vaccinia at 3×10^6 PFU per ml exhibited a morphologic progression similar to that of uninfected macrophages, with no evidence of cell clumping or damage. After 96 hr in culture the cell morphology was identical to that of uninfected macrophages (Fig. 7). Macrophages infected at 3×10^8 PFU per ml however, exhibited a greater tendency to clump and some evidence of cellular damage was observable 24 hr post infection (Fig. 8). By 48 hr post-infection there was a wide spread viral toxic effect which resulted in cell damage and death (Fig. 9).

Vaccinia Replication in Mouse Macrophages

To confirm that the mouse macrophage would not support replication of the vaccinia strain used in this study, virus

Figure 5: Mouse peritoneal macrophages after 2 hr in culture (X400). May Grunwald-Giemsa stain.

Figure 6: Mouse peritoneal macrophages after 96 hr in culture displaying extended morphology (X 400). May Grunwald-Giemsa stain.



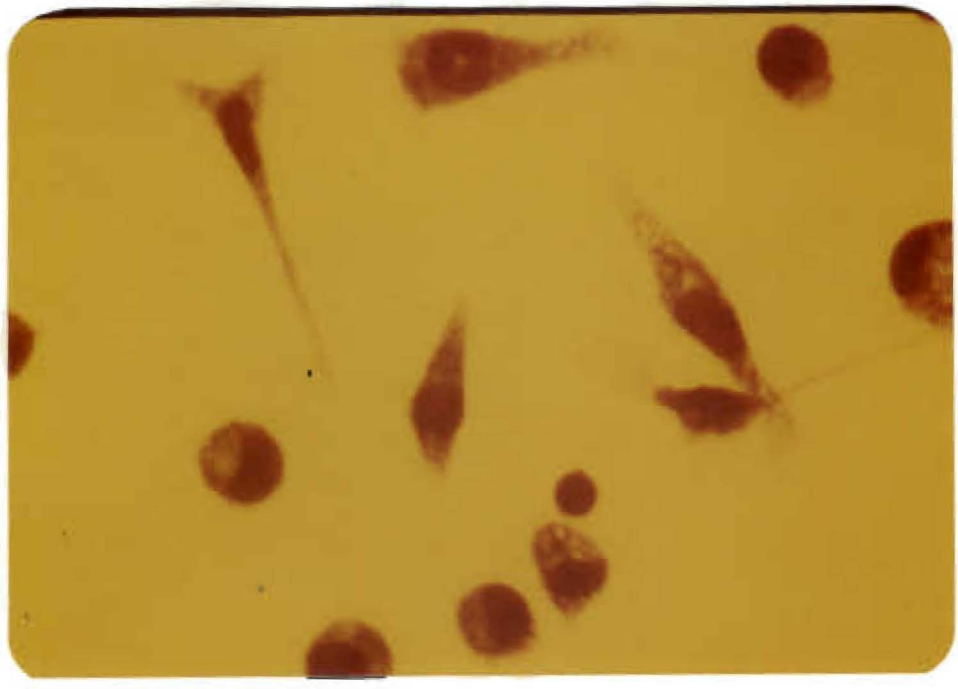
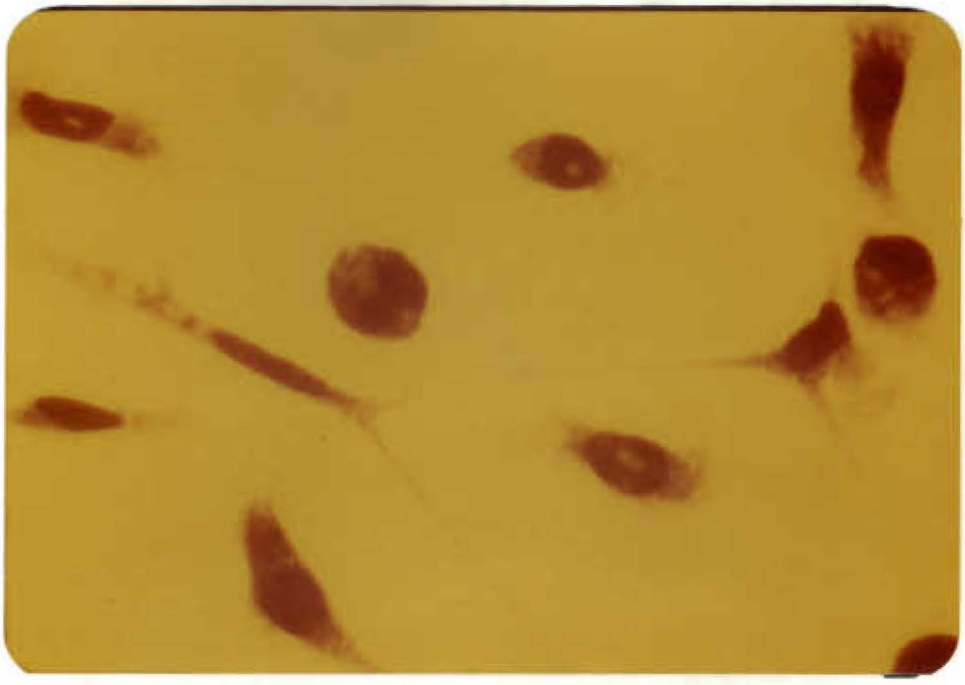


Figure 7: Mouse peritoneal macrophages infected with vaccinia at MOI of 1.0 for 96 hours (X 400). May Grünwald-Giemsa stain.

Figure 8: Mouse peritoneal macrophages infected with vaccinia at MOI of 100 for 24 hours (X 400). May Grünwald-Giemsa stain.



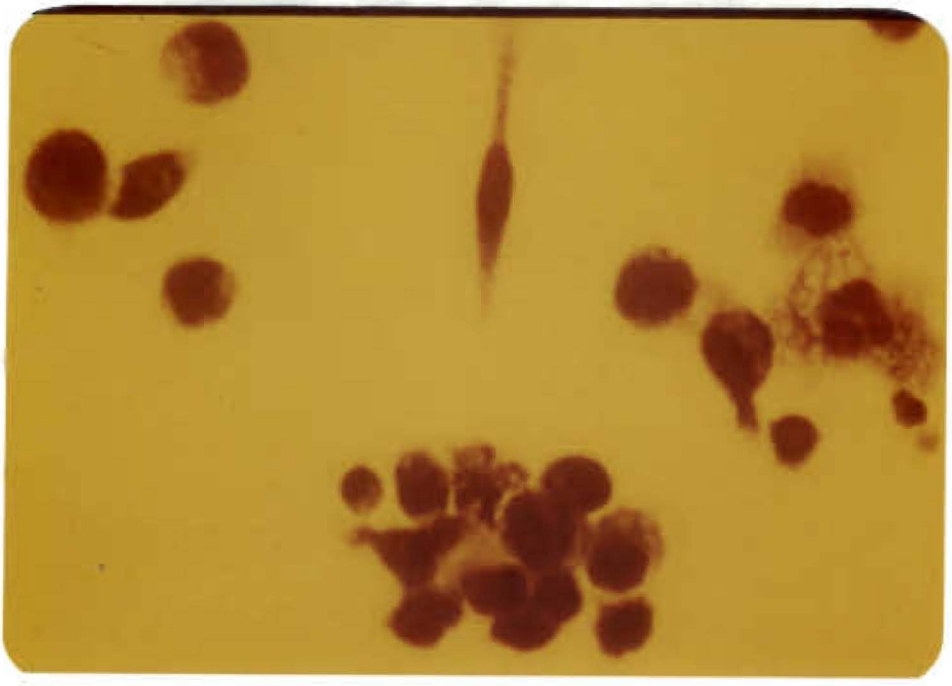
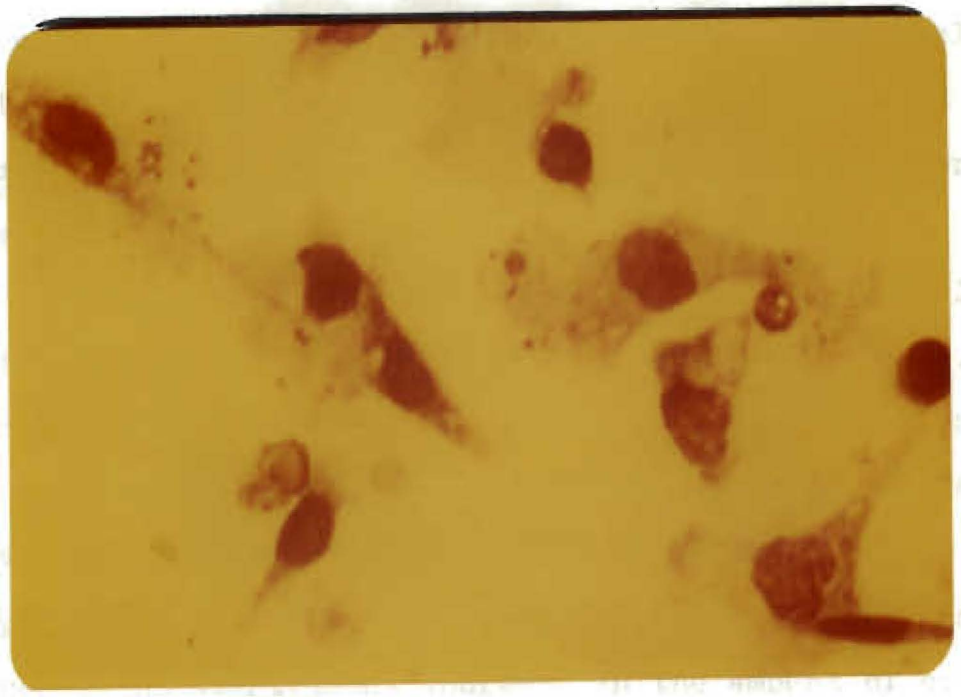


Figure 9: Mouse peritoneal macrophages infected with vaccinia at MOI of 100 for 48 hours (X400). May Grünwald-Giemsa stain.



was added to macrophages in coverslip tubes at 3×10^6 PFU per ml. Samples were assayed for viral replication and no increase in infectious viral titer was found through 96 hr post-infection (Fig. 10).

Autoradiography of Infected Macrophages

Although there appeared to be no formation of mature, infectious virus within the mouse macrophage, it was not known at which point during the viral replicative cycle the replicative block occurred. Autoradiography was used to determine whether the replicative block occurred before or after viral DNA synthesis.

The amount of discreet cytoplasmic silver-grain labeling, indicative of vaccinia DNA synthesis (Fig. 11 and 12), was found to be somewhat greater in the infected cells than in the uninfected cells (Fig.13). However, the maximum amount of vaccinia-like labeling found in the infected cells was only 1.4% of the total infected cell population. In addition, there was no significant increase in the amount of cytoplasmic labeling present in the infected cells through 72 hr as would be expected if viral DNA synthesis were occurring within infected cells. This lack of appreciable cytoplasmic labeling indicated that the block in viral replication occurred at a point earlier in the replicative cycle than DNA synthesis.

Second Stage Uncoating Assay of Infected Macrophages

In the replicative sequence of vaccinia, DNA synthesis

Figure 10: Growth of vaccinia in mouse peritoneal macrophages.

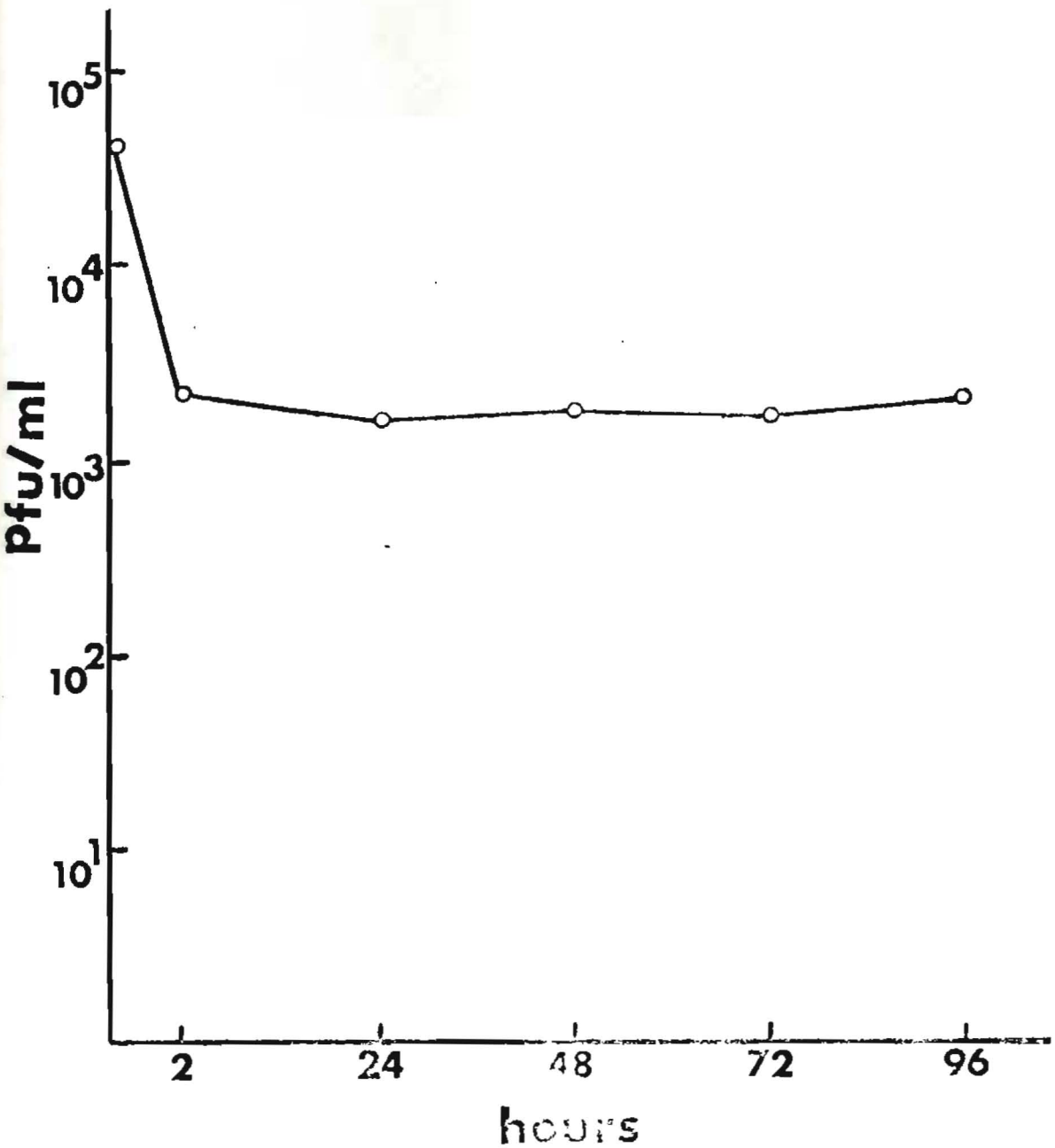
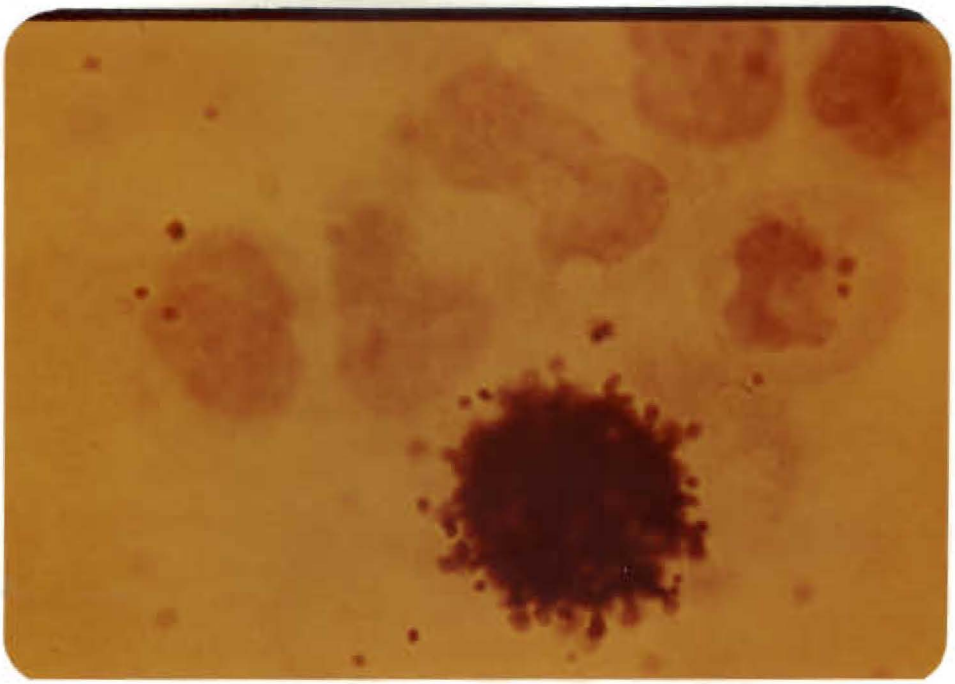

10^5
10^4
10^3
10^2
10^1
Pfu/ml
2
24
48
72
96
hours

Figure 11: Autoradiograph of uninfected mouse peritoneal macrophages displaying non-viral nuclear labeling which is due to cellular nucleic acid synthesis (X 1000).

Figure 12: Autoradiograph of mouse peritoneal macrophages infected with vaccinia at MOI of 1.0 displaying vaccinia-like labeling (X 1000).



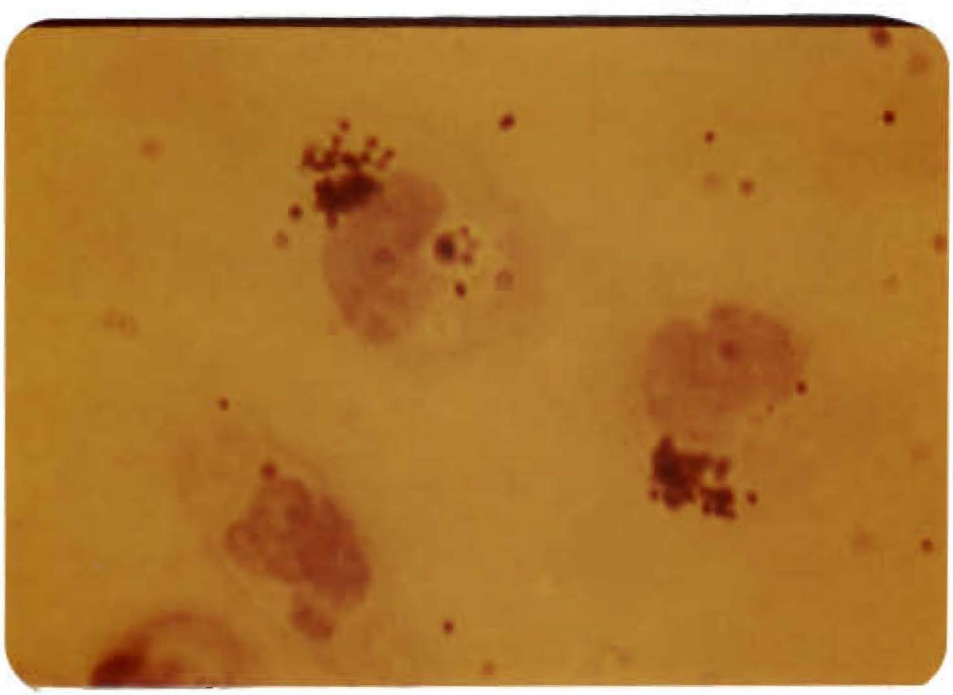
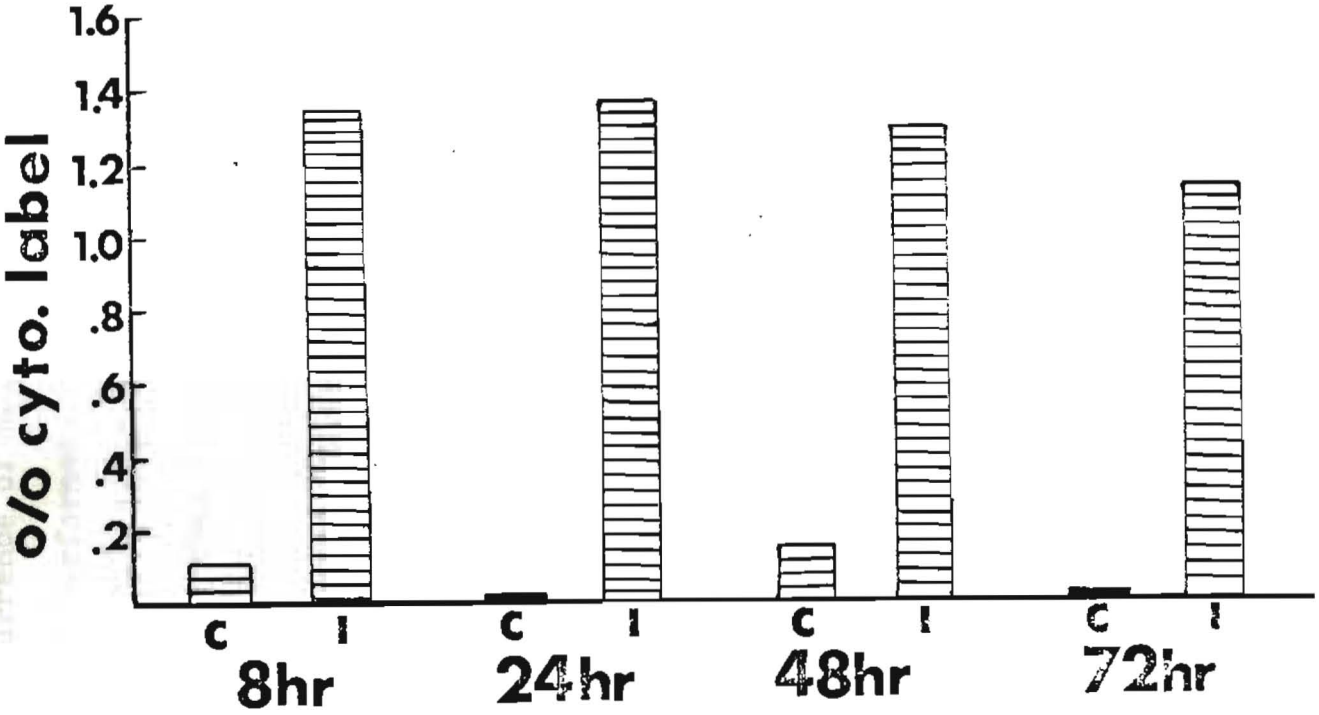


Figure 13: Comparison of viral induced cytoplasmic labeling in infected and uninfected control mouse peritoneal macrophages. Data obtained from 5000 cells counted per time period.


% cyto. label
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48hr
72hr

must be preceded by the release of the viral DNA from the protective core as a result of second stage uncoating. To test for the occurrence of second stage uncoating an uncoating assay was performed. The test involved infecting macrophages with virus labeled with ^{3}H-thymidine. The infected cells were then lysed by sonification. The lysate was then incubated with either DNase or buffer as a control, and the sample prepared for counting in a scintillation counter. Because only intact, acid-insoluable DNA was preserved by this technique, the amount of radioactive label which remained in the sample after DNase incubation was considered to represent that amount of viral DNA which was not susceptible to degradation by DNase and therefore had not been released from the core.

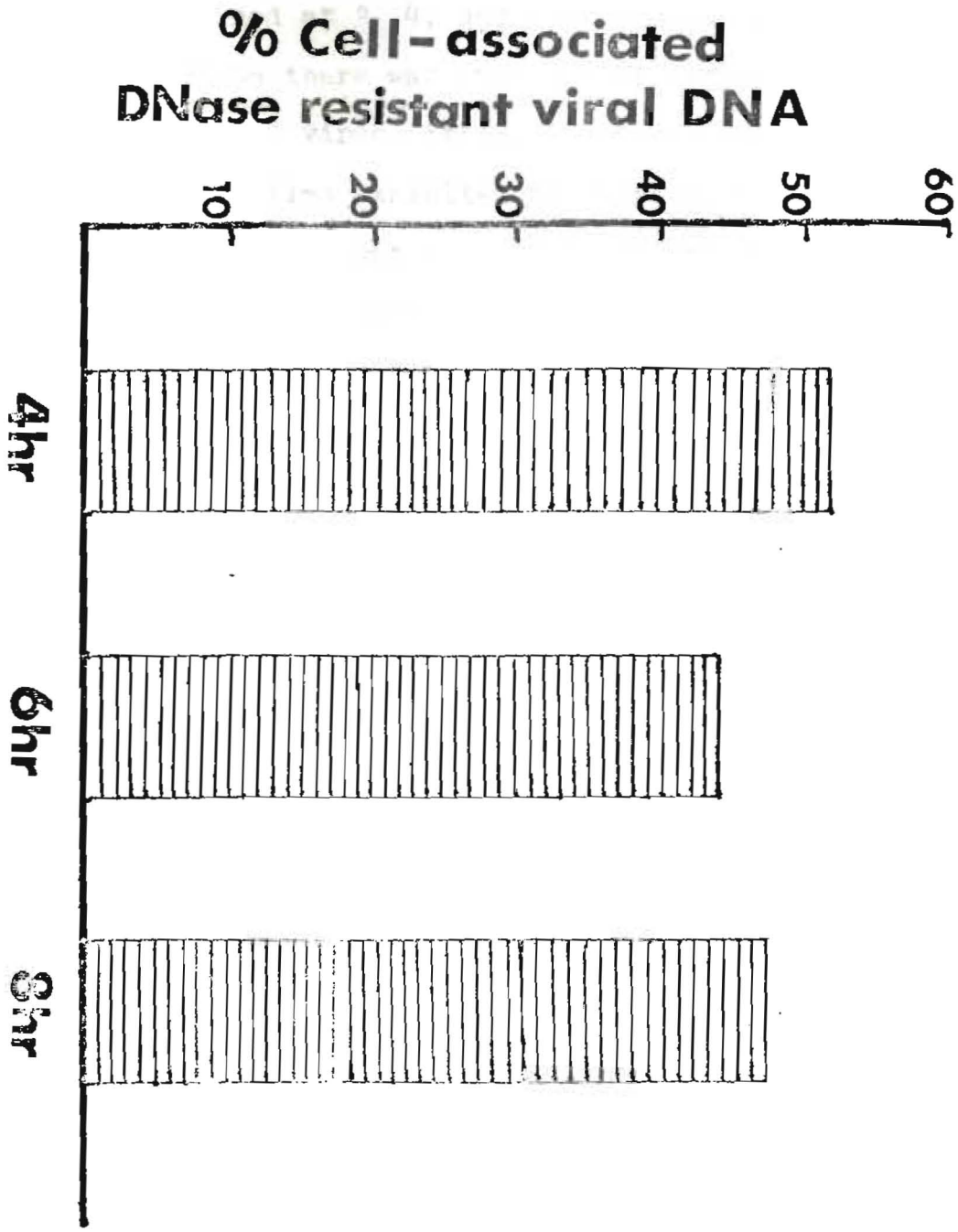
Approximately one half of the labeled viral DNA was lost upon exposure to DNase at 4, 6, and 8 hr post-infection (Fig. 14). There appeared to be no substantial increase in the amount of DNase-sensitive viral DNA with time, a result contradictory to that expected if the virus were undergoing second stage uncoating and releasing an increasing amount of free DNA into the cellular cytoplasm.

Electron Microscope Observations of Infected Macrophages

(1) Observations of Infected Mouse Macrophages

The electron microscope was utilized to further study the intracellular fate of vaccinia within the mouse macrophage.

Figure 14: Amount of cell-associated DNase-resistant viral DNA present within infected mouse peritoneal macrophages.


% Cell-associated
DNase resistant viral DNA
10
20
30
40
50
60
4hr
6hr
8hr

Macrophages were infected with 3 X 10^8 PFU per ml, an inoculum found to produce sufficient infected sections for study, and examined at 2, 4, and 6 hr post-infection. Two hr after infection there was evidence of both extracellular virus (Fig. 15) and virus within phagocytic vacuoles (Fig. 16). The virus at this time exhibited the typical viral morphology with a distinct outer coat and a dark staining, dumbbell shaped inner core (Fig. 17). Observations of cell sections at 4 and 6 hr after infection revealed virus still within phagocytic vacuoles. In opposition to the results reported by Silverstein (1970) in which he noted core formation by vaccinia in the mouse macrophage, no evidence of core formation or the occurrence of any later viral replicative form was observed in any of the sections examined from approximately 500 different infected cells. However, at 6 hr post-infection approximately one forth of the sections examined did contain virus within a phagocytic vacuole. At this time, the majority of the virus particles within the phagosome exhibited a diffuse, light staining outer periphery surrounding an indistinct darker staining region which had lost the characteristic core appearance (Fig. 18, 19, and 20). In addition, circular, virus-like particles similar to those described by Dales (1965) in studies involving lysomal degradation of inactivated vaccinia were observed (Fig. 21 and 22). In general, the virus contained within the phagosome 6 hr after infection had lost the typical mature morphology and appeared as a diffuse, atypical particle of variable

Figure 15: Electron micrograph of extracellular vaccinia virus (V) in close proximity to a mouse peritoneal macrophage (M) (X 190,000). Lead citrate stain.

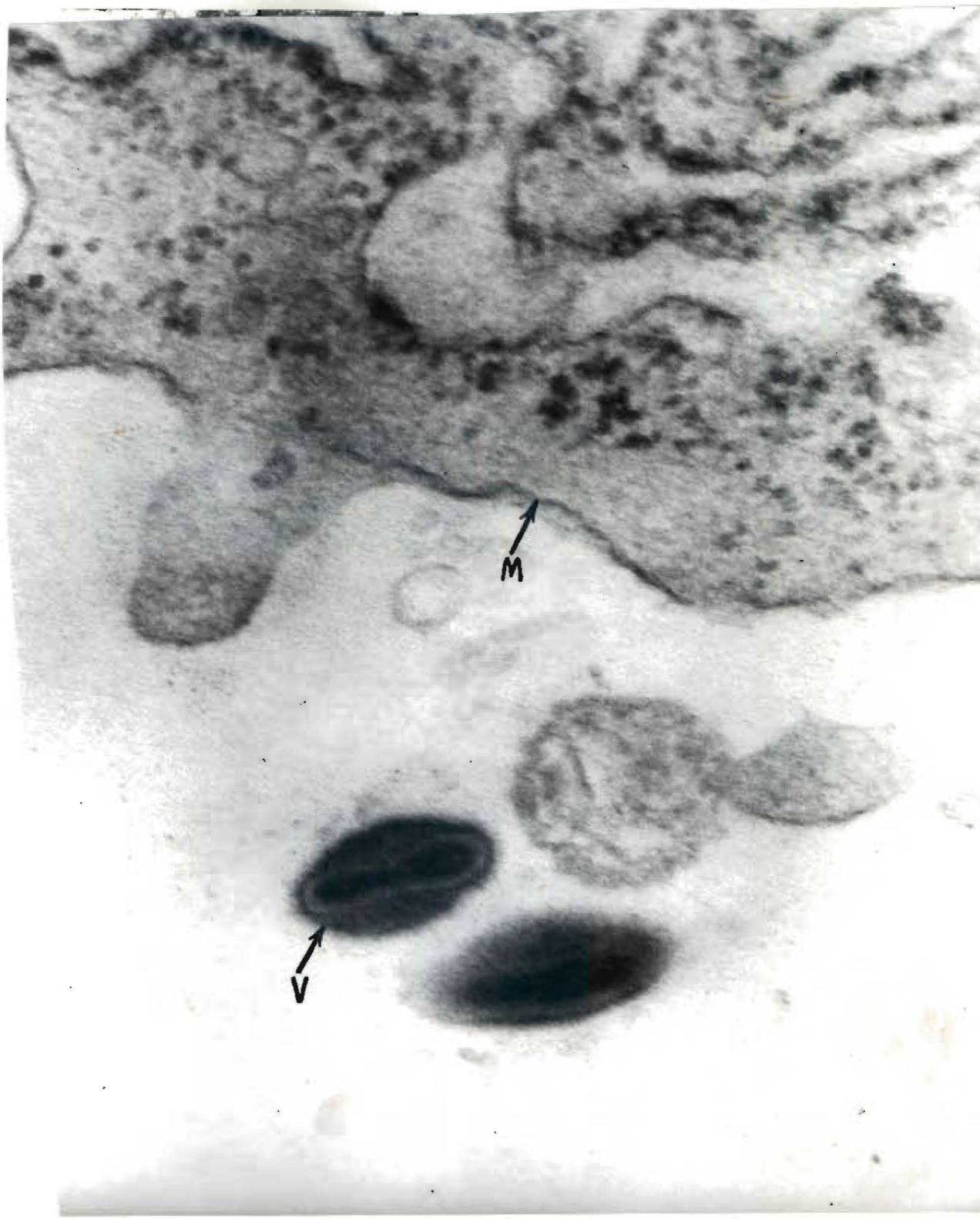

M
V

Figure 16: Electron micrograph of a mouse peritoneal macrophage containing vaccinia (V) within a phagocytic vacuole (PV) 2 hr post-infection (X 130,000). Lead citrate stain.

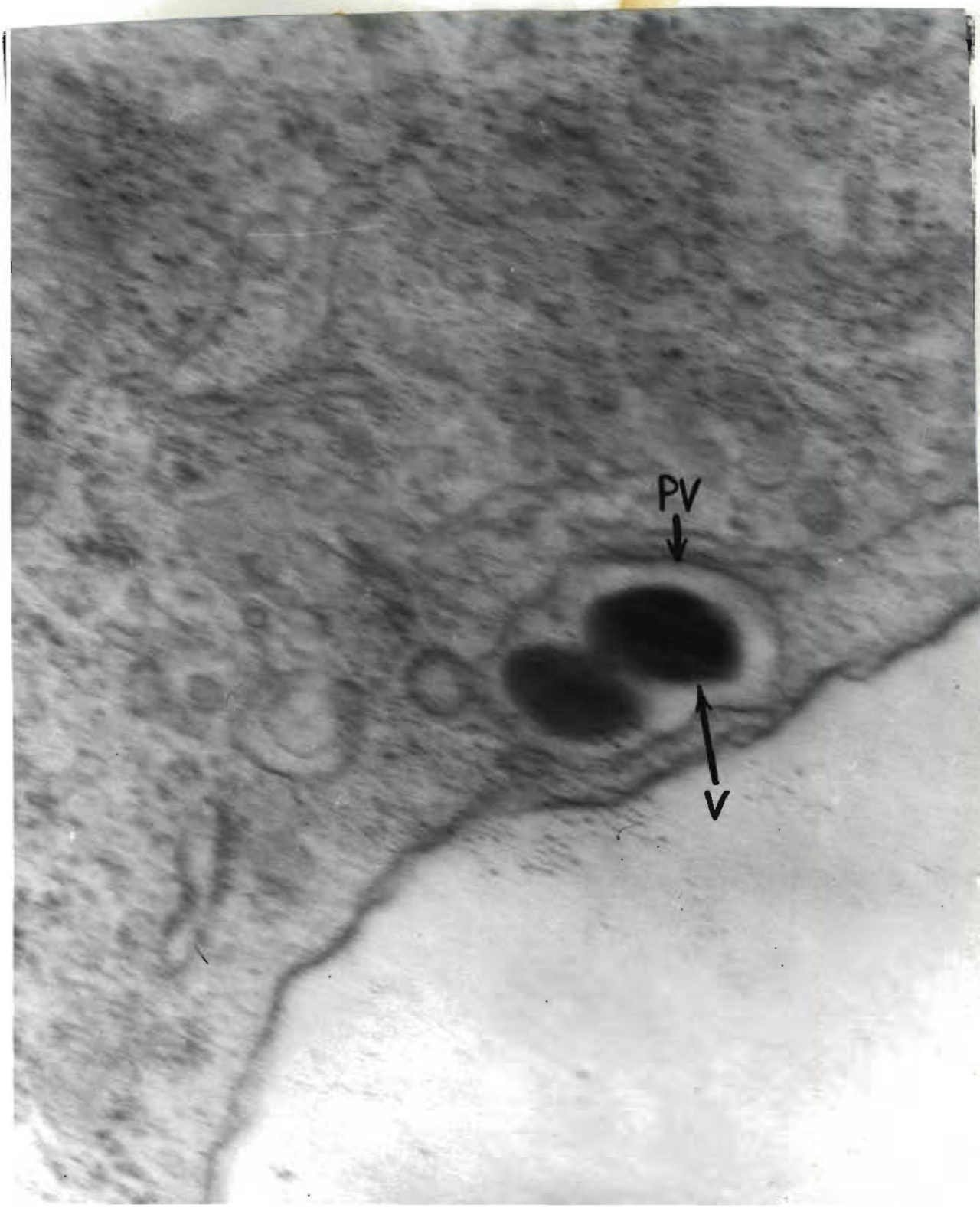

PV
V

Figure 17: Electron micrograph of vaccinia virus showing the typical outer coat (OC) and inner core (IC) morphology (X 225,000). Lead citrate stain.

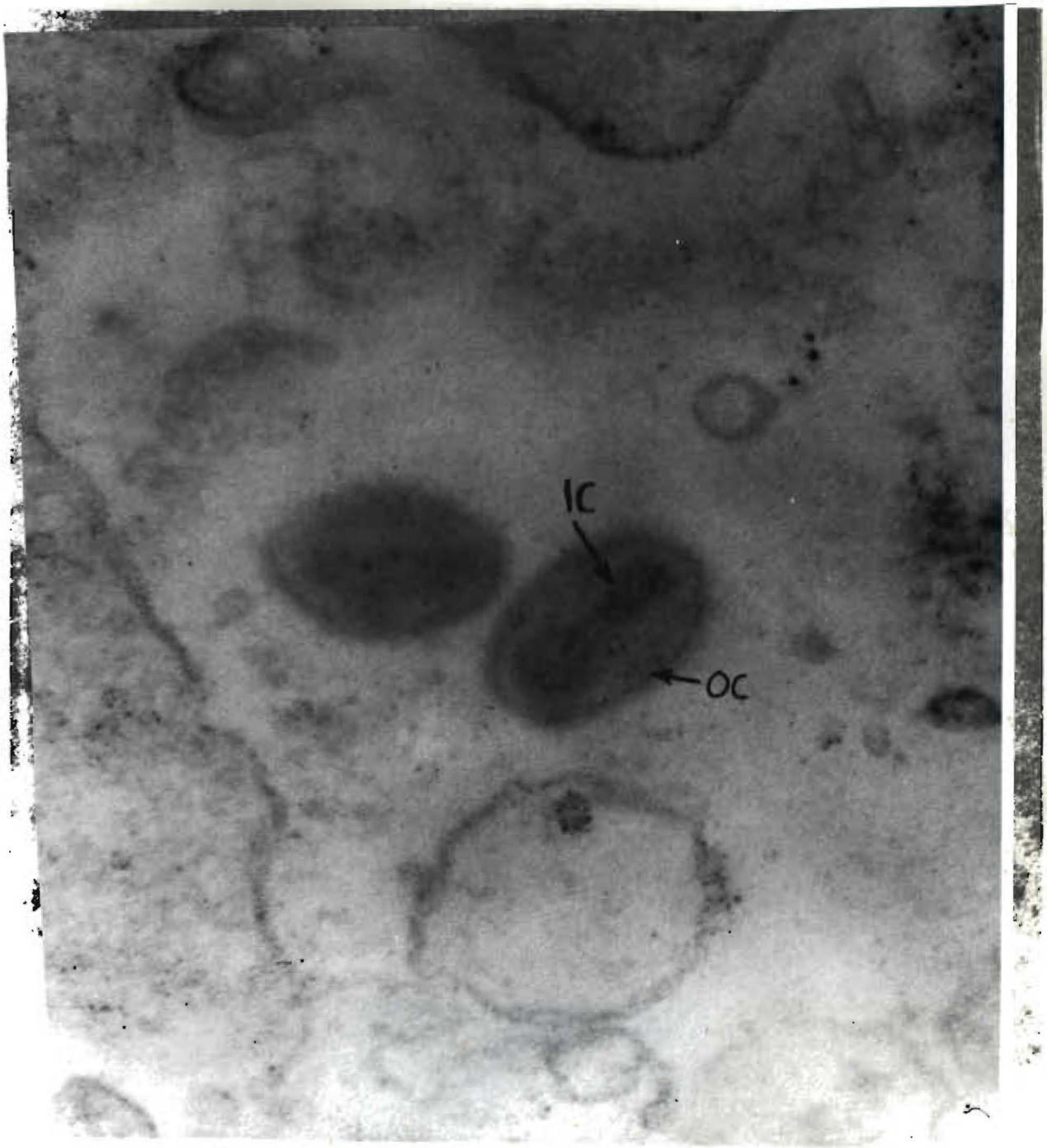

IC
OC

Figure 18: Electron micrograph of a mouse peritoneal macrophage containing vaccinia displaying abnormal morphology 6 hr post-infection. Particles B, C, and D all display an abnormal diffuse outer and inner appearance. Particle A displays the typical vaccinia morphology. (X 69,000). Lead citrate stain.

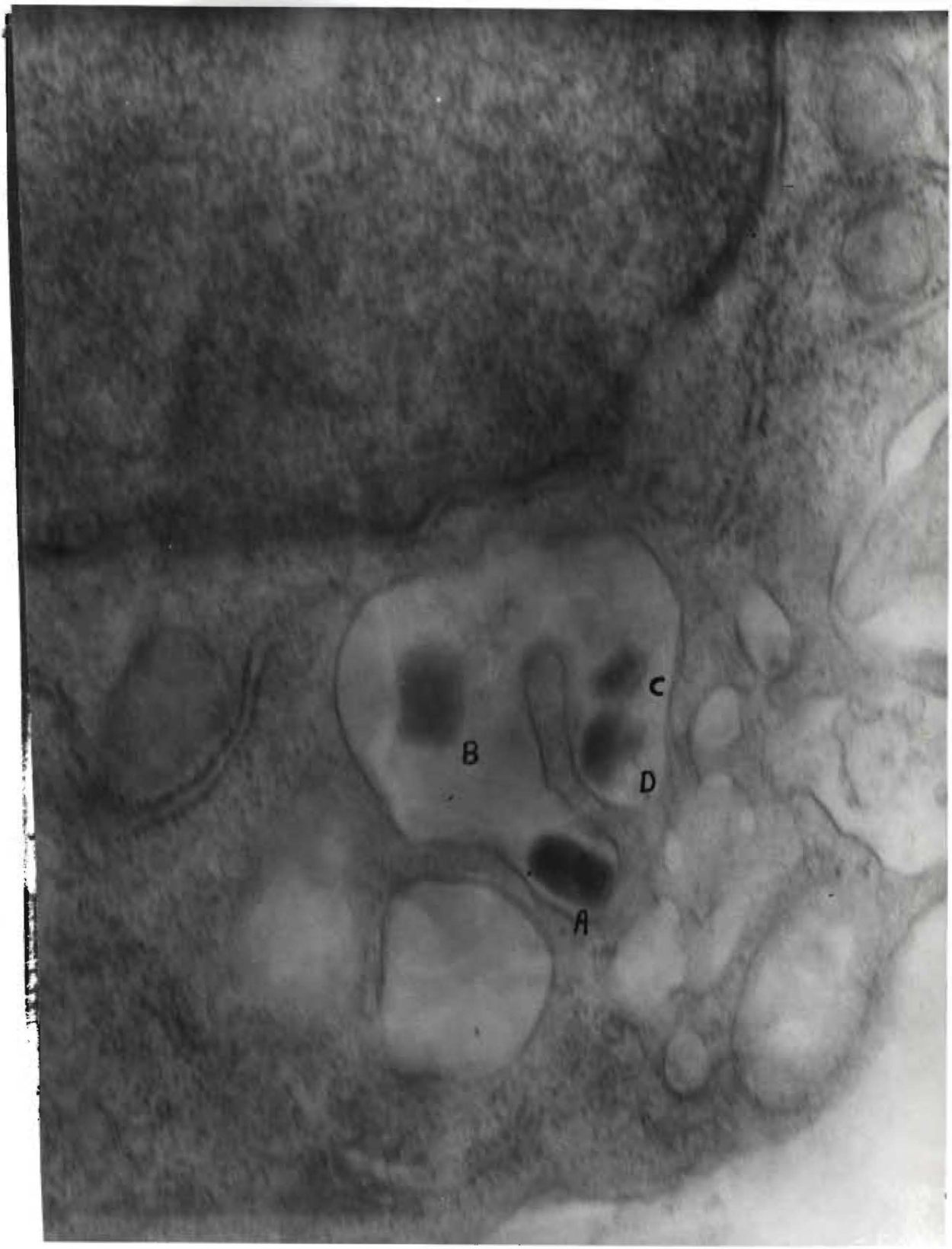

C
B
D
A

Figure 19: Electron micrograph of a mouse peritoneal macrophage containing a virus particle (V) which displays an abnormal diffuse morphology 6 hr post-infection (X 64,000). Lead citrate stain.

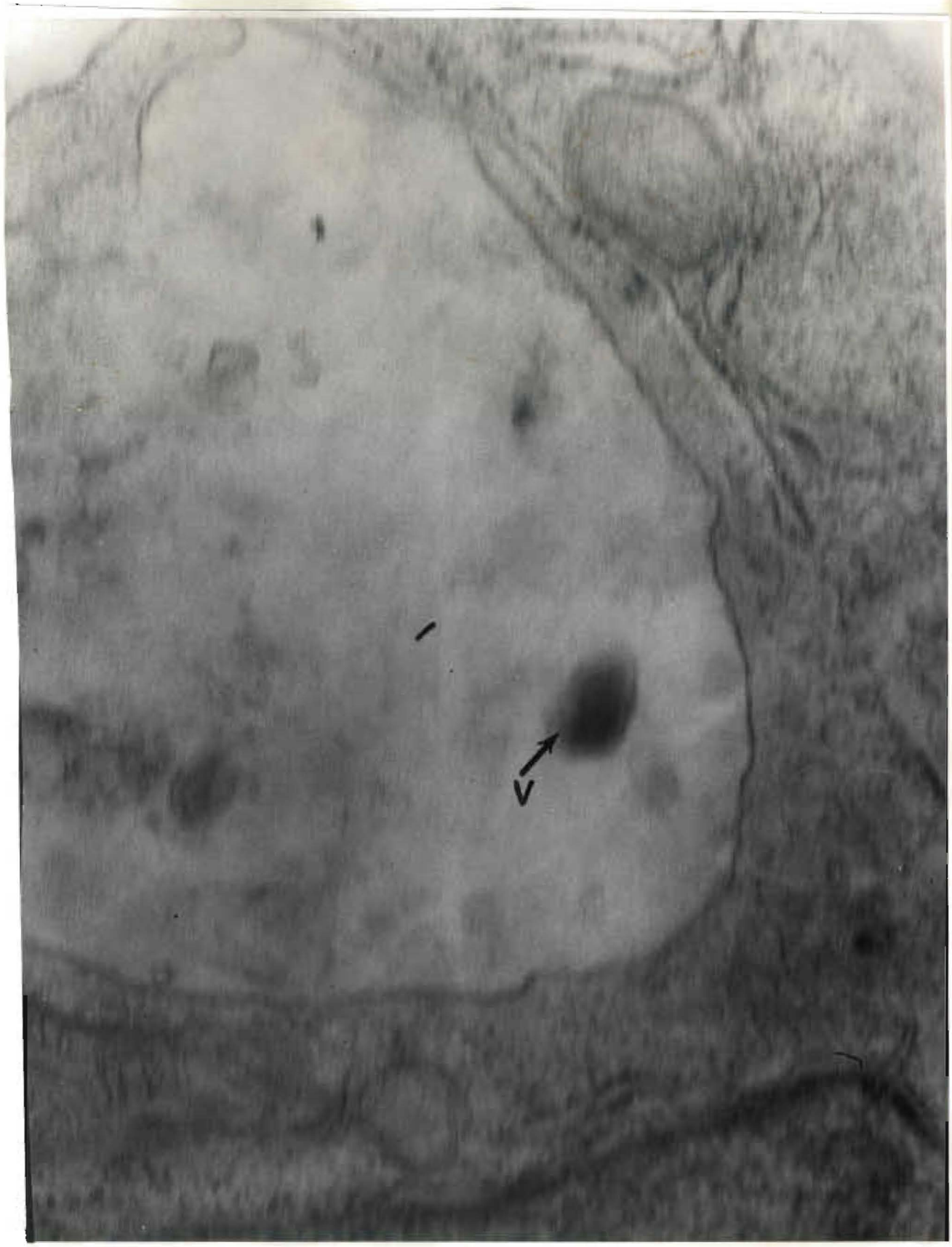

V

Figure 20: Electron micrograph of a mouse peritoneal macrophage containing a virus particle (V) displaying an abnormal diffuse morphology 6 hr post-infection (X 53,000). Lead citrate stain.

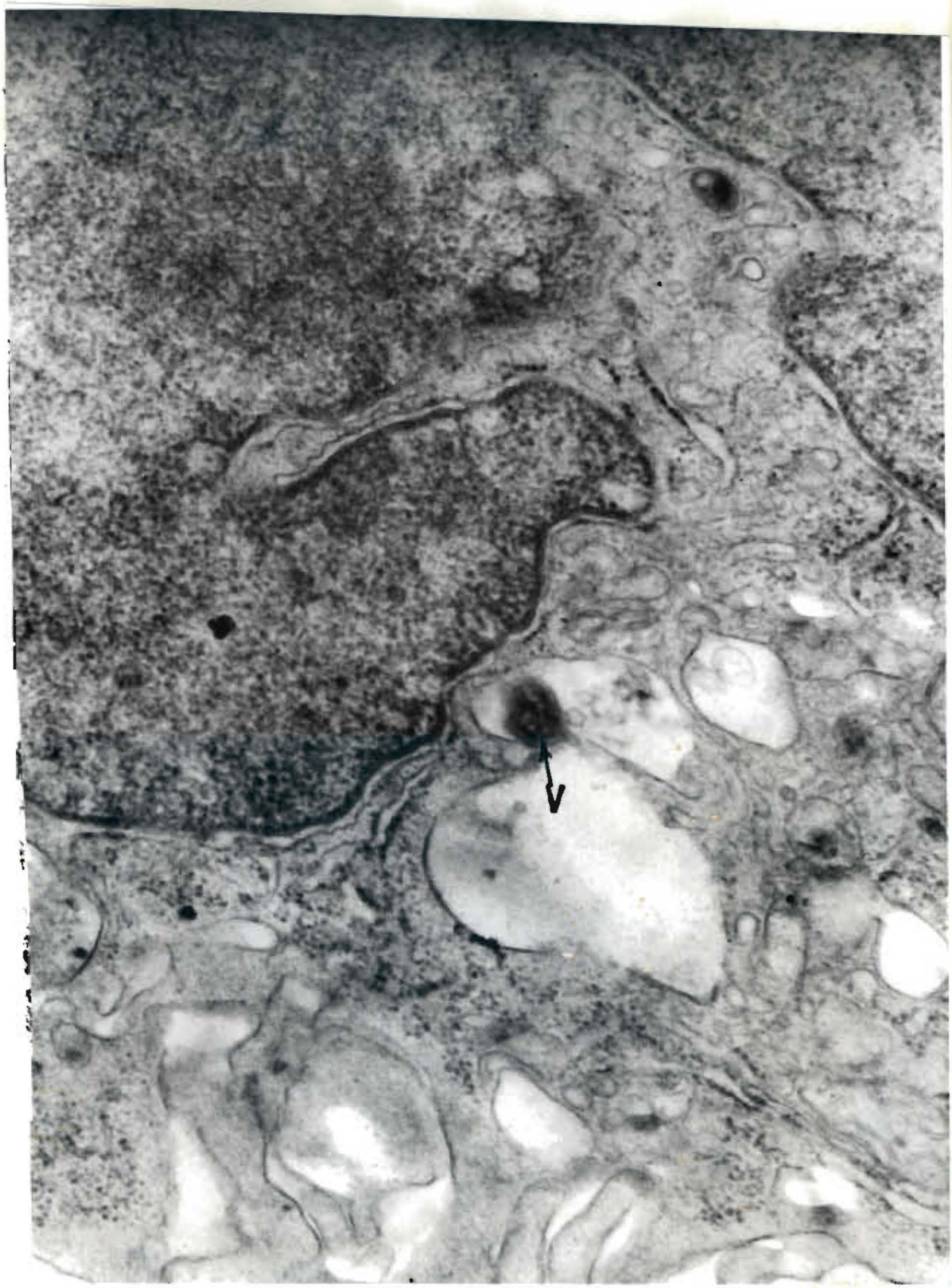
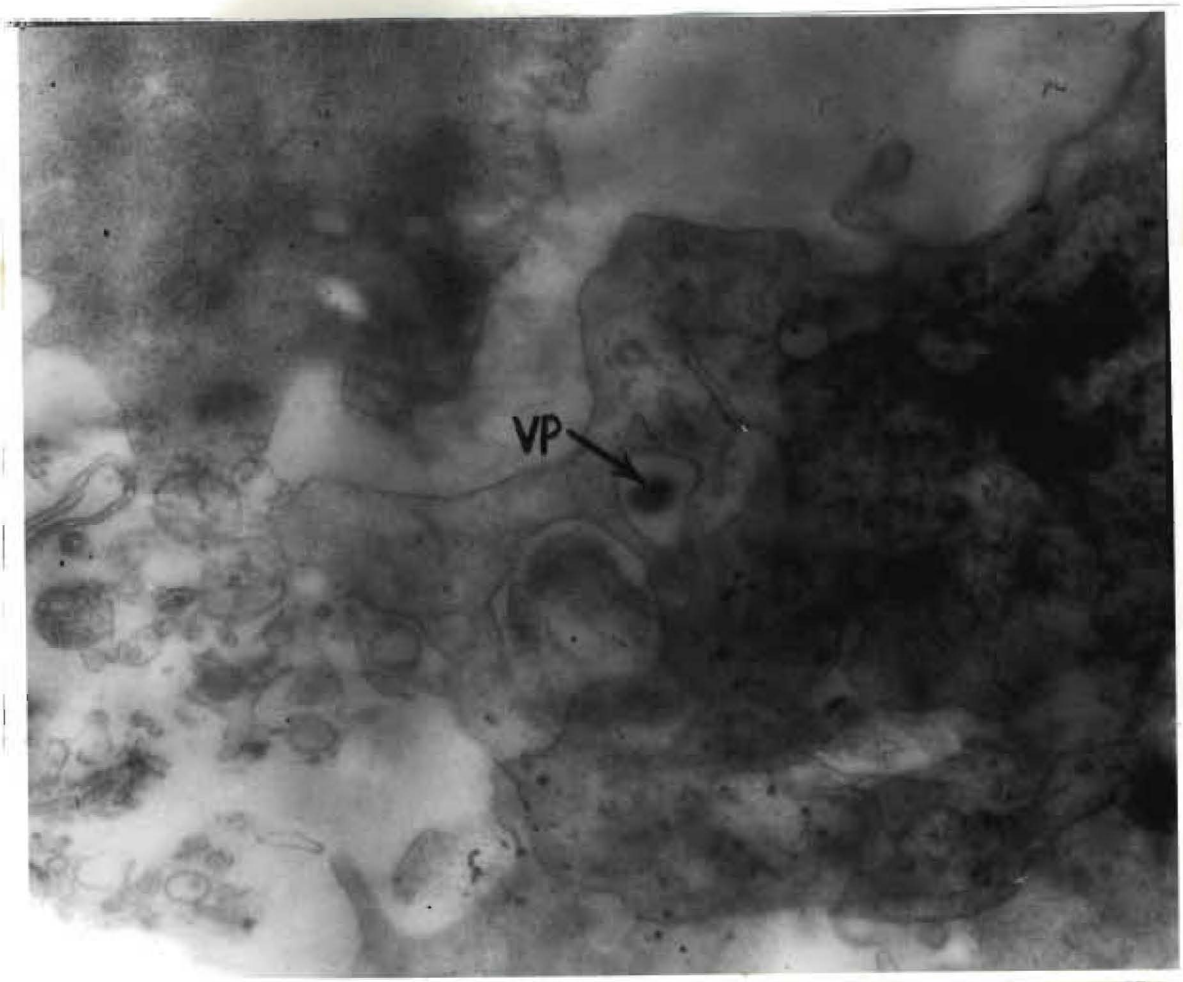
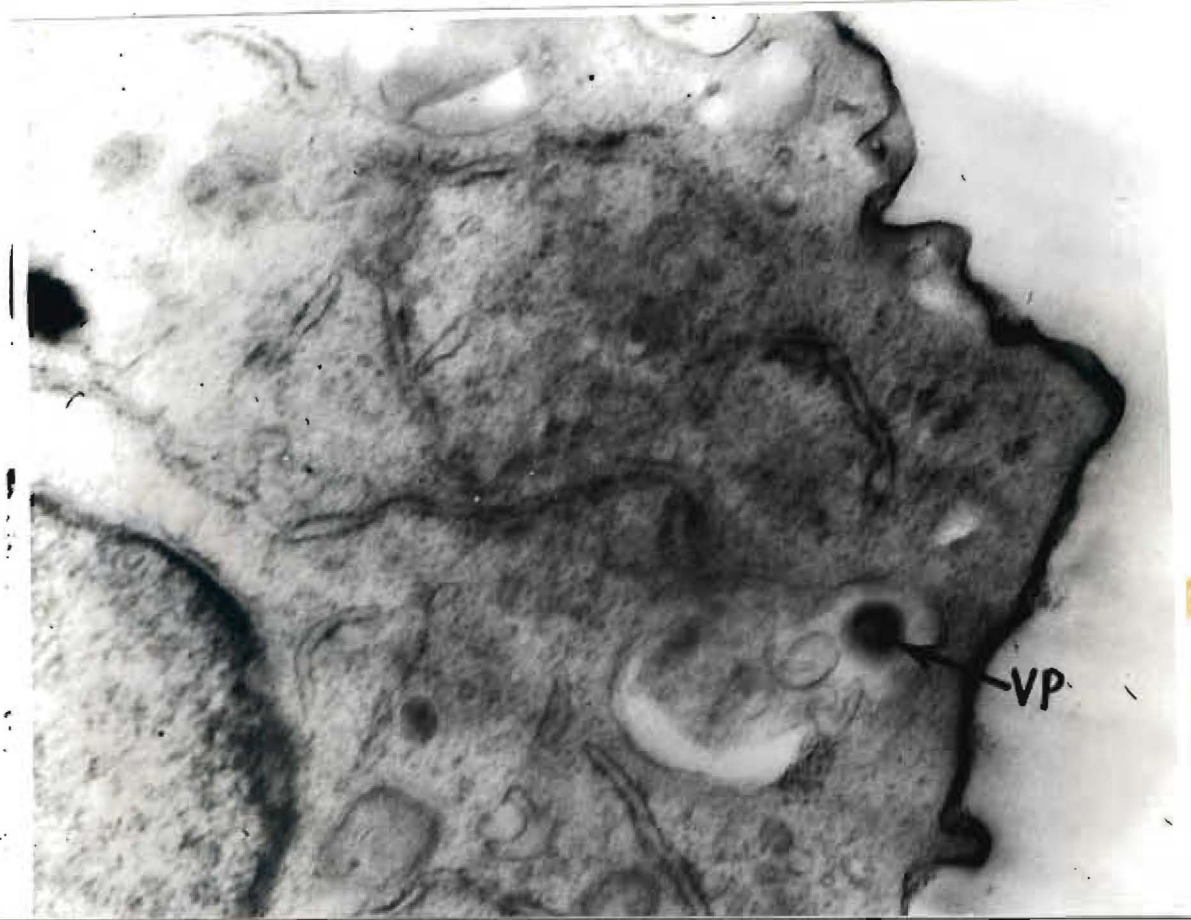


Figure 21 and 22: Electron micrographs of mouse peritoneal macrophages containing virus-like particles (VP) 6 hr post-infection.
(Fig. 21 = X 29,000)
(Fig. 22 = X 39,000)
Lead citrate stain.


VP


VP

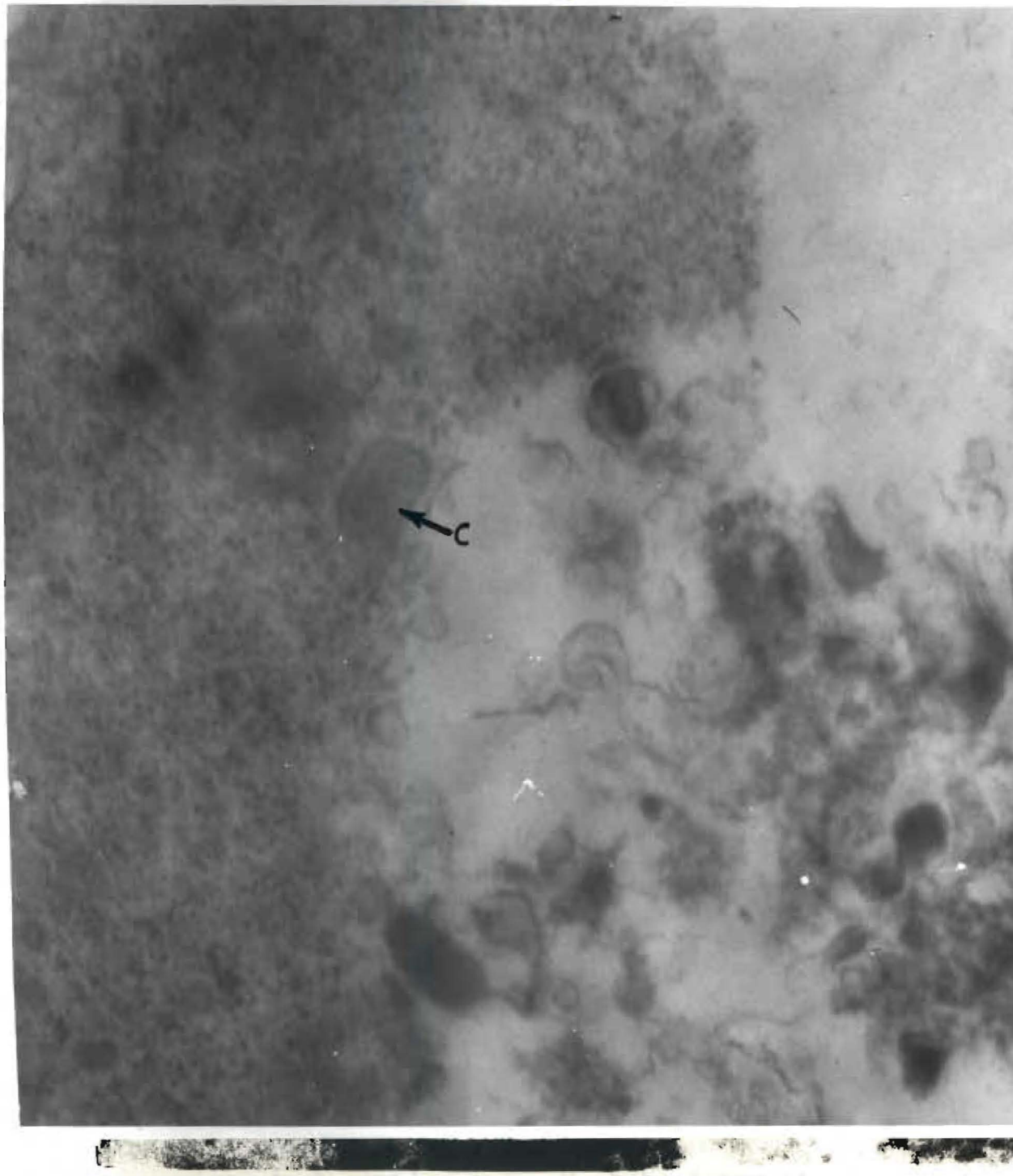
size and shape. This abnormal viral morphology was taken to indicate that lysomal degradation of the virus within the phagocytic vacuole was in progress.

(2) Observations of Infected Rabbit Macrophages

In order to confirm that vaccinia would form cores observable by the electron microscope techniques used in this study normal rabbit peritoneal macrophages were infected with vaccinia at 1.5×10^8 PFU per ml and examined for the presence of cores. Viral core formation was observed in the infected rabbit macrophages 3 hr after infection (Fig. 23).

Thus, infected mouse peritoneal macrophages were found to block the replication of vaccinia at a point before the occurrence of first stage uncoating in accordance with the results of Schultz (1966).

Figure 23: Normal rabbit peritoneal macrophage containing a vaccinia core (C) 3 hr post-infection (X85,000). Lead citrate stain.


C

DISCUSSION

Because macrophages are situated in all the major body mpartments, it is inevitable that these cells will encounter fecting viral particles, usually at an early stage of fection. Much evidence now suggests that in many cases e success and severity of a viral infection depends upon is initial virus-macrophage encounter (Mims 1964; Blandon 70).

There appears to exist two basic types of macrophage sistance to viral infection. One type is that which curs naturally, is genetically inherent, and does not pend upon an acquired immune response to the virus. The cond type of macrophage resistance is that which develops rough the process of acquired cellular immunity.

The naturally occurring resistance of mouse macrophages vaccinia infection was first reported by Nishmi and rnkopf (1958) and confirmed by Nishmi and Niecekowski 963). These workers reported that mouse peritoneal crophages, when infected *in vitro* with vaccinia, failed support viral replication.

Beard and Rous (1938) established that rabbit peritoneal crophages were permissive for vaccinia replication while mpkins *et al*. (1970) and Avila *et al*. (1972) showed that ritoneal macrophages from rabbits immunized with vaccinia uld not support viral replication. Work by Greer *et al*. 974) demonstrated that vaccinia was phagocytized and derwent at least first stage uncoating within peritoneal

macrophages from immunized rabbits. Buchemeier et al. (1979) established that vaccinia replication is halted during the viroplasmic condensation stage late in the viral replicative cycle within the immune rabbit macrophage. The exact mechanism whereby the assembly of new viral particles is inhibited has not been determined to date.

Other studies on the interaction of vaccinia with the mouse macrophage have yieled somewhat conflicting results. Schultz (1966), using fluorescent antibody techniques, reported the disappearance of labeled viral antigen following macrophage infection and suggested that vaccinia was degraded by the lysosomal enzymes within the phagocytic vacuole.

Silverstein (1970), in an electron microscope study, observed viral cores within infected mouse macrophages although biochemical techniques revealed that viral DNA uncoating had not occurred. Cell death followed several hours after infection and was attributed to a cytopathic effect. Silverstein suggested that vaccinia replication was halted after first stage uncoating in the mouse macrophage with subsequent cell lysis.

However, Mackaness and Raffel (1971) reported that vaccinia was inactivated and partially degraded within the phagocytic vacuole of mouse macrophages with no core formation occurring. The membrane of the phagosome was observed to break down and release the virion into the cell cytoplasm which led to cell death, presumably from a toxic effect.

Thus, there appears to be some question as to the manner which vaccinia replication is inhibited. The present udy was undertaken with the goal of further examining e interaction of vaccinia with the mouse macrophage.

It was found that stimulated mouse macrophages from n-immune animals, after infection at low multiplicities, d not support vaccinia replication. In contrast to the sults of Silverstein (1970) and Mackaness and Raffel (1971), fected macrophages exhibited no evidence of cellular nage after prolonged culture. Although no increase in rus was found in the infected systems, neither was there appreciable decline in the amount of cell-associated fectious virus. Because the macrophage cultures were tained from peritoneal exudates which contained a mixed ll population, some adherent fibroblast-like cells may t have been removed by the washing procedure and were fected, yielding some new virus. This may partly explain e finding of virus within the system 96 hr after infection. e more likely possibility is that vaccinia, although it es not replicate, is held within the macrophage in an fectious form for an extended time, probably within the agocytic vacuole.

Results of an autoradiographic study of infected mouse crophages revealed a small increase in vaccinia-like toplasmic labeling in infected cultures as compared to infected controls. This finding may be due to the fact at macrophages, following ingestion of foreign particles,

display an increased metabolic activity which includes an increase in mitochondrial nucleic acid synthesis. Such synthesis areas would be expected to incorporate radioactive label which would appear in the cell cytoplasm similar to that of vaccinia-labeling. It should be noted that the maximum amount of infected cells demonstrating cytoplasmic labeling was only 1.4% of the total infected cell population and there was found to be no increase in the amount of cytoplasmic labeling with time. In similar experiments utilizing permissive cells which allow viral DNA syntesis to occur, the amount of viral-induced cytoplasmic labeling increases with time until almost 100% of infected cells contain such label (Matthews, 1978; Shidani, 1980). The lack of progressive cytoplasmic labeling in the vaccinia infected cultures is interpreted as an absence of DNA synthesis in the mouse macrophage.

Although autoradiography indicated viral DNA synthesis was not occurring, the results did not specify the point of inhibition. A study to detect the occurrence of viral DNA uncoating within infected mouse macrophages yielded results difficult to correlate with established findings. In contrast to Silverstein (1970), it was found that approximately one half of the DNA from cell-associated virus particles became DNase sensitive within the macrophage, suggesting that viral DNA uncoating had occurred. However, the kinetics of the uncoating process observed in this study were not in agreement with those reported by Joklik

63) due to the fact that there was no increase in the
unt of DNase sensitive viral DNA with time. These
ults may be partially explained by the presence in the
al inoculum of intermediate viral replicative forms
taining DNase-sensitive viral DNA which became cell-
ociated. These sub-viral forms occurred as a result
harvesting virus from infected mouse embryo cells. The
ld included not only fully mature virus but incomplete
ms as well. These particles, while not infectious,
ght be expected to contain labeled DNA. However, the
sence of DNase-sensitive viral DNA within the macrophage
represent the product of rapid but partial degradation
hin the phagocytic vacuole of the macrophage resulting
a stable viral form containing DNase-sensitive viral DNA.

An electron microscope study of infected macrophages
performed to determine whether viral replicative forms
e present in the cell cytoplasm or whether the virus
being degraded within the phagocytic vacuole. Macro-
ges infected with low multiplicities (less than MOI of
yielded electron microscope preparations in which it
difficult to find enough infected cell sections for
dy. To remedy this problem, macrophage cultures were
fected at a MOI of 100, a multiplicity which did not
pear to adversely affect the macrophages until approximately
24 hr after infection, at which time a toxic effect
noticealble. To confirm that early steps in vaccinia
plication would proceed at such a high multiplicity of

infection, normal rabbit peritoneal macrophages were infected at a toxic MOI of fifty. Viral cores were observed 3 hr after infection in the rabbit macrophages. These results are in accordance with those from a study by McGuire (1973), in which she reported the presence of late viral replicative forms within rabbit peritoneal macrophages infected with a toxic dose of vaccinia. These results suggested that the process of core formation within the mouse macrophage, if it were occurring, would not be inhibited by the size of the infecting inoculum. However, in contrast to the results reported by Silverstein (1970), examination of infected mouse macrophages revealed no evidence of viral replicative forms up to 6 hr after infection. Viral particles were observed within phagocytic vacuoles of mouse macrophages 6 hr after infection, a time well beyond that required for viral first stage uncoating to occur in other permissive and non-permissive cell types (Dales and Kajoka, 1964; Joklik, 1963; Greer et al., 1974; Matthews, 1978; and Buchmeier et al., 1979).

The majority of viral particles observed within phagocytic vacuoles of mouse macrophages 6 hr after infection appeared to be undergoing lysosomal degradation, as evidenced by their abnormal, diffuse morphology. Dales (1965) reported that vaccinia inactivated by heat, exposure to ultraviolet light, or absorption with specific antibody remained within the phagocytic vacuoles of infected cells in which it was slowly degraded by lysosomal enzymes. Virus-like particles,

milar to those described by Dales as being vaccinia
dergoing degradation, were observed in this study within
e phagosome of mouse macrophages 6 hr after infection.
cording to Dales, the failure of inactivated vaccinia
break out of the phagocytic vacuole was due to the in-
tivation of a component, probably proteinaceous, of the
ter viral coat which was necessary for the transfer of
e viral core into the cell cytoplasm. Similarly, Schultz
966) suggested that the resistance of mouse macrophages
vaccinia was due to the presence of hydrolytic enzymes
ich denatured the viral uncoating component and led to
e degradation of the virus particle within the phago-
tic vacuole. The failure of rabbit macrophages to
hibit vaccinia replication was attributed to the lack
such enzymes, the presence or absence of which would
controlled by the genetic constitution of the host cell.

The state of macrophage activation is also known to
gulate the antimicrobial activity of these cells.
tivated macrophages, as described by Mackaness (1964 and
69), show an increased phagocytic and antimicrobial
pability over unactivated macrophages. True macrophage
tivation occurs as a result of an immunologic stimulation
diated by lymphocytes, although Karnovsky and Lazdine
975) showed that macrophages stimulated with peptone
hibit very similar characteristics. These workers reported
at peroxidase, an enzyme known to be integral to the anti-
crobial activity of granulocytes (Klebanoff, 1968), was

present in high levels within stimulated mouse peritoneal macrophages. Further evidence that macrophage activation plays an important role in resistance to viral infection was obtained by Rama Rao et al. (1977). These workers reported that macrophages from homozygous nude mice raised in a conventional environment would not support viral replication, in contrast to macrophages from such mice raised under germ free conditions or reconstituted with thymic transplants which allowed viral replication. The resistance displayed by macrophages obtained from nude mice raised under conventional conditions was believed to be a function of activation resulting from chronic infections, rather than the type dependent on interacting T lymphocytes.

The role of interferon in macrophage resistance was also examined by Rama Rao et al. (1977). It was found that resistant macrophages from nude mice failed to produce significant amounts of interferon even after infection of the animals with vaccinia, thus demonstrating that interferon did not play a major role in the acquired macrophage resistance. These findings were in agreement with those of De Ckercq and DeSomer (1973), who reported that the in vivo resistance of mice to vaccinia which followed polyacrylic acid or Brucella abortus injection, was not due to increased interferon levels but rather to macrophage activation by these agents.

Thus, it appears that differences in the level of

crophage activation may be primarily responsible for the tcome of a macrophage-virus enounter. It seems reasonable assume that the conflicting results which have been ported in the literature involving the interaction of use peritoneal macrophages with vaccinia may have as eir basis the variable of macrophage activation. Electron croscopic observations made in the present study, which ilized stimulated macrophages, suggest that an activated use macrophage is a truly resistant rather than non-rmissive cell type, because vaccinia is unable to initiate s' replicative sequence within the cell. However, the servation that viral titer did not decrease within fected macrophages in vitro indicates that the virus was t completely inactivated but was contained in an infectious rm at least through 96 hr post-infection.

The results of this study lend further support to the portance of the macrophage in the in vivo protection of ult mice against vaccinia infection. It is postulated at the mouse peritoneal macrophage may exhibit varied grees of resistance to vaccinia due to the occurrence of sosomal enzymes, the presence or absence of which is ntrolled by the level of macrophage activation. Further udy is required to more fully understand this seemingly mplex interaction of macrophage physiology and host sistance.

SUMMARY

1. Stimulated mouse peritoneal macrophages were infected with vaccinia virus *in vitro* and found not to support viral replication. In addition, no viral-induced cell damage was observed at non-toxic multiplicities of infection.
2. An autoradiographic study of infected macrophages revealed that no significant viral DNA synthesis was occurring.
3. Infected macrophages were assayed for the occurrence of viral second stage uncoating. The results indicated that free viral DNA was present within the macrophage. It was suggested that this DNA was probably due to viral degradation and not a replicative process.
4. Electron microscopic examination of infected macrophages revealed the presence of virus within phagocytic vacuoles 6 hr after infection. The majority of this virus appeared to be undergoing lysosomal degradation as evidenced by the abnormal viral morphology.
5. The results of this study indicate that the stimulated mouse peritoneal macrophage is a resistant cell type in regards to vaccinia infection. This resistance is at least partially controlled by the level of macrophage activation.

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LITERATURE CITED

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