FACTORS AFFECTING UPTAKE AND REPLICATION OF VACCINIA VIRUS IN PERITONEAL MACROPHAGES IN VITRO

A Thesis

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by

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

The mononuclear phagocytes, which comprise the reticulo-endothelial system (RES), represent a large, widely distributed and morphologically heterogeneous group of cells. They are involved in diverse functions related in large measure to their endocytic activities and to the intracytoplasmic digestion which is a consequence of their heterophagic function.

Monocytes and fixed macrophages of the RES play an important role in host susceptability to, and against viral pathogens. Macrophages are located in all the major compartments of the body. Those lining the liver, spleen, and marrow sinusoids monitor the blood and remove, for instance, effete red cells. Those lining the sinuses of lymph nodes monitor the lymph removing microorganisms or inert particles brought in by the afferent lymph stream. Those lining the pleural and peritoneal cavities monitor these cavities; and those lining the respiratory tract monitor the respiratory fluid film, ingesting inhaled dust particles, mineral particles, or microorganisms.

Virus particles, it is suggested, tend to be taken up by macrophages as are other foreign particles. Since these cells effectively monitor the main body compartments, they inevitably encounter infecting virus particles at an early stage in infection.

Although many properties of the mononuclear phagocytes are relatively clear, there are a number of aspects concerning their ultrastructure, life history, synthetic capabilities, and relationships to other cells in the immune system which have remained obsecure. The factors responsible

for the changes which occur in the structure and function of macrophages during an infectious disease are not precisely known. There seems no doubt, however, that an immunological process is involved (Mackaness, 1964). It is also true that we know very little about the machinery that controls the reactivity of their plasma membrane and that influences their role as effective mediators of host defense.

The fate of virus particles within elements of the RES has been studied only within the past thirty-five years. The poxvirus-macrophage interaction has been investigated more extensively than the interaction of any other virus with this cell type. The peritoneal macrophages are easily obtained from experimental animals and thus have been studied more than other macrophages.

The ability of poxviruses to replicate in macrophages appears related to both virus and cell strain. Beard and Rous (1938) have shown replication of vaccinia virus in cultured rabbit macrophages, while Nishmi and Niecikowski (1963) have demonstrated the failure of vaccinia to multiply in mouse macrophages. It, therefore, seemed of interest to study these virus-macrophage interactions in vitro to determine the effects of some factors on this relationship.

HISTORICAL BACKGROUND

Eli Metchnikoff in 1884 presented the theory of cellular immunity to infectious disease. His view was that immunity was dependent upon certain body cells called phagocytic cells. While studying the water flea (Daphnia) he observed that phagocytic cells could ingest and destroy yeasts (Monospora bicuspidata) that were pathogenic to the flea. Metchnikoff spent the rest of his life studying the phenomenon of phagocytosis in higher animals.

Emil von Behring studied the bactericidal quality of blood for several different pathogenic microbes. From these studies arose the theory of humoral immunity to infectious disease. He stated in 1890 that immunity to diphtheria and to tetanus was dependent upon the capacity of blood to inactivate diphtheria or tetanus exotoxin. Wright and Douglas in 1904 published convincing evidence that both cellular and humoral factors played a part in immunity.

The cellular and humoral theories of immunity have been greatly expanded over the years. Some of the advances in cellular immunity will be reviewed in this section. Lymphocytes and macrophages are the major cell types contributing to the cellular immune response. Emphasis has been placed on the macrophages of the RES.

The autoradiographic studies of Volkman and Gowans (1965) have identified the bone marrow as the principal site for blood monocytes. Similar studies have demonstrated that tissue and peritoneal macrophages are derived from circulating monocytes. The macrophage appears intimately associated with many of the most important pathways of immunity. Delayed

hypersensitivity, induction of the primary antibody response, and clearance of foreign proteins all have been associated with macrophage function.

The role of cellular and humoral factors in susceptability to viral infections is a subject of continuing investigation. The existence of a relationship between phagocytes and defense against viral infections has been recognized and studied only in the past thirty-five years. The poxvirus-macrophage interaction has been studied more extensively than that of any other virus.

Vaccinia virus on cultivation in vitro with Kupffer cells or macrophages of rabbits was shown to replicate (Beard and Rous, 1938), while vaccinia virus failed to multiply in mouse peritoneal macrophages (Nishmi and Niecikowski, 1963). The mouse macrophage cultures reacted in a unique manner by exhibiting a progressive decrease in infectious viral titer. This extended a previous observation on the diminishing titer of vaccinia virus in macrophage cultures from random-bred mice (Nishmi and Bernkopf, 1958). The rabbit and mouse macrophage-virus interactions correlate with the fact that vaccinia virus causes a non-lethal infection in rabbits, but has no apparent effect upon adult mice (Zakay-Roness, 1962).

Immunization of rabbits with infectious vaccinia virus was shown to have a different effect on the response of various tissues to subsequent in vitro challenge with the same virus (Steinberger and Rights, 1963). Cultures of kidney cells obtained from immunized or nonimmunized rabbits yielded similar quantities of virus when infected with vaccinia virus. In contrast, cultures of spleen cells obtained from immunized

rabbits exhibited an impaired capacity for supporting multiplication of vaccinia virus in comparison with controls.

The presence of resistance in "immune" spleen but not kidney cultures suggested to the workers that this resistance was in some way associated with the macrophages, which were more abundant in the spleen cultures. This view was supported by diminution of resistance in spleen cultures challenged with vaccinia virus seven to eight days after seeding; when owing to a rapid growth of fibroblastic cells, there was a relative decrease in macrophages.

Neutralizing antibody in the "immune" spleen cultures was not detected despite the use of a relatively sensitive plaque neutralization method. The virus-adsorbing capacity of the "immune" spleen cells was found to be similar to that of the nonimmune spleen cells. Therefore, it was concluded that the cells did not contain surface antibody.

The observation that patients with aggamaglobulinemia readily recovered from many viral infections was made by Gitlin (1959). The fact that these patients could be successfully immunized against smallpox suggested that cellular factors rather than the formation of antibodies may be of prime importance in viral resistance.

Tompkins, Zarling, and Rawls (1970) examined the possible contributions of lymphocytes and macrophages to immunity to vaccinia virus in vitro by determination of antigen-specific inhibition of macrophages and the replication of the virus in alveolar and peritoneal macrophages. Enhanced cellular immunity parallels delayed hypersensitivity in almost every virus disease. An in vitro correlate to the delayed hypersensitivity reaction, which develops upon immunization with vaccinia, is the

capillary migration test of George and Vaughn (1962). The test is based on the characteristic of macrophages to migrate from capillary tubes over a glass surface. Migration of peritoneal exudate cells from sensitized animals is inhibited if the sensitizing antigen is present. The inhibition results from a specific interaction between antigen and sensitized macrophage (Bennet and Bloom, 1967).

Peritoneal exudate cells from immune animals were inhibited from migrating in the presence of vaccinia virus antigens but were not inhibited from migrating by other virus antigens (Tompkins, Zarling, and Rawls, 1970). Another source of macrophages was tested. Alveolar macrophage preparations were examined for their migration characteristics in the presence of vaccinia virus antigens. Unlike the peritoneal exudate cells, the alveolar macrophages were not inhibited from migrating in the presence of viral antigen.

Results of their experiments demonstrated that vaccinia virus was capable of replicating in peritoneal macrophages from nonimmune rabbits but not in peritoneal macrophages from immune animals. This immunity was not observed in alveolar macrophages. Vaccinia virus replicated readily in alveolar macrophages from both immune and nonimmune rabbits. The interpretation of these results was that the difference reflected the route of immunization or could represent basic differences in phagocytic cells from the two sources. The alveolar macrophages are thought to originate within the lung interstitium (Bowden et al., 1969), where they develop biochemical characteristics that distinguish them from other types of macrophages.

Further work was done on vaccinia virus specific macrophage immunity by Avila, Schultz, and Tompkins (1972). Virus adsorption studies showed the fraction of cells adsorbing virus and the total virus adsorbed to be the same for immune and nonimmune macrophages which was in agreement with Steinberger and Rights (1963). Subsequent events in the virus replication cycle differed. Infective center studies revealed that essentially all of the macrophages in the normal cell population were susceptible to virus infection. In contrast, the infection of cultures of immune macrophages did not appear to spread from cells originally adsorbing virus.

Results of neutralization of virus by immune serum of virus infected immune macrophages lead these authors to the conclusion that vaccinia was fixed at the immune cell surface and remained accessible to neutralizing antibody. There has been no previous evidence to suggest retention of particulate antigens at macrophage membranes (Unanue and Cerotinni, 1970).

Other poxviruses include the fibroma-myxoma group of rabbit viruses, which are closely related to one another, but only distantly related to the vaccinia-variola group. Myxomatosis virus and macrophage interaction has been investigated. Ginder (1955) showed that rabbit macrophages could support the replication of myxomatosis virus. The virus-neutralizing activity of macrophages from fibromatosis virus immune and susceptible rabbits was also measured. Virus neutralization was assayed by measuring the size of tumor that developed following subcutaneous injection of virus-cell mixture into a susceptible rabbit. Monocytes and

peritoneal, spleen, and liver macrophages from immune animals exhibited greater neutralizing ability than did the combination of hyperimmune serum and cells from susceptible animals. The neutralizing ability was shown to be virus specific.

In contrast to vaccinia, ectromelia or mousepox, another type of poxvirus, can be grown in mouse macrophages (Roberts, 1964). Roberts studied the interaction of virulent and avirulent strains of ectromelia virus with mouse peritoneal cells and demonstrated that macrophages were more susceptible to infection with the virulent than with the attenuated viral strain. Macrophages obtained from mice immunized against ectromelia were more readily infected than were normal macrophages. This effect was attributed to the greater phagocytic activity of macrophages from immune mice. Even in the presence of immune serum, macrophages from immune mice were more readily infected than were macrophages from normal mice.

There are susceptible strains and genetically resistant strains of mice to ectromelia. Differences in virus growth in the two strains of mice appeared at a certain stage of infection, which was the time at which neutralizing antibody was demonstrable in the blood of resistant mice (Schnell, 1960). In this case it appeared that humoral factors were responsible for the animals' resistance, as macrophages from susceptible and resistant strains of mice were equally susceptible to infection in vitro with virulent ectromelia virus.

Factors affecting viral replication or the lack of replication in macrophages have not been fully investigated. Therefore, it was of

of interest to this author to investigate conditions and variables in the replication of vaccinia virus in peritoneal macrophages.

MATERIALS AND METHODS

Virus

Two strains of vaccinia virus were used for this study. One strain was originally obtained from smallpox vaccine points produced by Eli Lilly and Company. The second was the IHD strain of vaccinia virus obtained from American Type Culture Collection of Animal Viruses, Rickettsiae and Chlamydiae.

Tissue Culture Medium

Eagle's Minimum Essential Medium (MEM) containing L-glutamine plus combiotic (penicillin, 200 units per ml; and streptomycin, 100 units per ml) and serum varying in concentration from 5 to 30% depending on cell type being cultured, and adjusted to pH 7.0 with 10% sodium bicarbonate was used as culture medium for all cells.

Chicken fibroblast cells were grown in medium supplemented with 5% bovine serum. The serum content was reduced to 2% for titration or growth of virus on monolayers.

Rabbit macrophages were harvested in Hanks' Balanced Salt Solution (BSS) plus heparin (two USP units per ml) and combiotic, and adjusted to pH 7.0 with 1.4% sodium bicarbonate. After settling of cells the Hanks' BSS harvesting medium was removed and replaced with complete Eagle's MEM culture medium plus combiotic and 10% (bovine or fetal bovine) or 20% (normal or immune rabbit) serum.

Mouse macrophages were harvested in heparinized Hanks' BSS, settled,

and the medium replaced with Eagle's MEM plus combiotic and 30% bovine serum.

Sera

Bovine serum was prepared using whole blood obtained from freshly slaughtered animals at the Fanestil Packing Co., Inc., Emporia, Kansas. After clotting, serum was decanted and centrifuged at 2500 rpm for ten minutes to clarify, filtered through a bacteriological filter (HA 0.45 u), aliquoted, and frozen. Before use the serum was heat inactivated at 56° C for 30 minutes in a water bath.

Sterile fetal bovine serum was obtained from Colorado Serum Company and heat inactivated before use.

Rabbit serum (normal or vaccinia immune) was obtained by collecting blood aseptically from the marginal ear vein or directly from the heart of the appropriate rabbit. The serum was decanted after clotting, centrifuged, collected, and heat inactivated. Immune serum was tested by viral neutralization before use.

Propagation of Virus

Virus was propagated on chicken embryo fibroblast monolayer cultures. Nine to eleven day old chicken embryos were removed from eggs and placed in 1X GKN (saline solution containing potassium ions and glucose). The embryos were decapitated, eviscerated, and washed. Fragmentation of embryos into single cells was accomplished by compressing the remains through a glass tipped syringe, then trypsinizing for one hour in 0.2% trypsin (1:250, General Biochemicals) dissolved in 1X GKN. After washing

the chicken embryo cells were resuspended in Eagle's MEM plus 5% bovine serum plus combiotic at a concentration of 2 x 10^6 cells per ml then planted 50 ml per Roux bottle. The cells were incubated at 37° C for 24 to 48 hours until the monolayer was nearly complete. The medium was removed, the monolayer washed once with Delbecco's Phosphate Buffered Saline (PBS) and infected with 10^7 plaque forming units (PFU) of virus in 10 ml of PBS plus 0.1% bovine serum albumin (BSA). The virus was allowed to adsorb to the cells for two hours at 37° C with occasional agitation of bottles to prevent the monolayer from drying out. The adsorption medium containing unadsorbed virus was removed, and 50 ml of Eagle's MEM plus 2% bovine serum and combiotic was replaced per infected monolayer. Monolayers were returned to incubation until extensive cellular destruction was evidenced (24 to 48 hours).

Harvesting of Virus

When the production of virus was complete Roux bottles were frozen, cell side covered with medium, at -70° C. Then the Roux bottles were removed from the freezer and allowed to thaw with frequent agitation to loosen remaining cells from the glass surface. A 50 ml volume of propagated virus was placed in the treatment unit of a Raytheon Sonic Oscillator Model DF-101 and sonified at maximum intensity for one minute to release virus from cell debris and break up clumps of virus. Any remaining debris was removed by centrifugation of the sonified virus fluid for 10 minutes at 3500 rpm. Supernatants were aliquoted in appropriate amounts and frozen at -70° C until used.

Assay of Virus

One ounce Duraglas prescription bottles planted with 3 ml of 2 x 10^6 chicken embryo cells/ml in Eagle's MEM plus serum and combiotic were used for titration of samples of virus after a monolayer had formed (48 hours). The medium was removed, and monolayers were washed once with Delbecco's PBS. Dilutions of virus sample were made in PBS plus 0.1% BSA. One ml of virus dilution was added per monolayer using duplicate dilutions of the appropriate range. Virus was allowed to adsorb to the monolayer for two hours at 37° C, then the unadsorbed virus remaining in the dilution fluid was removed. Two ml of Eagle's MEM plus 2% bovine serum and combiotic was added to each monolayer. Monolayers were incubated at 37° C for 36 to 48 hours for plaque formation. After removal of medium the monolayers were stained with 2 ml of a 1:10 dilution in distilled water of Gram's crystal violet for two to four minutes. The stain was removed and the monolayer washed twice with tap water. The plaques present on countable dilutions were counted. Virus activity was calculated and expressed as plaque forming units (PFU) per ml.

Experimental Animals

New Zealand white rabbits three to five months of age were obtained from breeders in Emporia and the surrounding area. The rabbits were used at the age of six to eight months.

White mice, descendants of a strain obtained from Kansas State University, Manhattan, Kansas, were bred and raised in the Emporia State Microbiology animal room. The mice used were young adults.

Immunization Procedure

Four to six intradermal injections, 0.1 ml each, were made on the shaved back of rabbit with a virus suspension of 3×10^7 PFU/ml. After 14 to 21 days, when the lesions had healed, three or four intravenous injections of two ml of 10^8 PFU/ml of virus were given at intervals of six days. Five or six days after the last injection, rabbits were bled and/or macrophages were harvested.

Harvesting of Rabbit Peritoneal Macrophages

Experimental rabbits were injected intraperitoneally with 50 ml of sterile mineral oil (Squibb Heavy Duty) five days prior to harvest. Animals were sacrificed either by injection of 20 - 30 ml of air into the marginal ear vein or by exsanguination by bleeding from the heart. The abdominal hair was saturated with Purasan and shaved. The skin layer was opened exposing the muscle sheath, 100 ml of Hanks' BSS plus heparin were injected into the peritoneal cavity, and the abdomen was massaged. The cavity was opened, and the fluid removed with a sterile 50 ml volumetric pipette and emptied into a separatory funnel. The funnel containing the exudate fluid was placed at 4° C for ten minutes to allow the oil to separate, leaving the cells in the Hanks' BSS layer. The cell layer was drained into 50 ml polycarbonate centrifuge tubes and centrifuged at 1500 rpm for ten minutes. The supernatant was removed, the cells washed with fresh Hanks' BSS and recentrifuged at 1500 rpm for ten minutes. The supernatant was removed, and the cells were resuspended in a known volume of Hanks' BSS. A cell count was taken with a Neubauer hemacytometer. The cell suspension was

adjusted to 3×10^6 cells per ml with Hanks' BSS. One ml was planted per tube in flying coverslip tubes. The tubes were incubated at 37° C for one hour at an angle allowing the cells to become attached to the coverslips. The Hanks' BSS was removed and the cells were washed once with fresh Hanks' BSS. Complete culture medium, Eagle's MEM plus serum and combiotic, was placed on the cells.

Harvesting of Mouse Peritoneal Macrophages

Experimental mice were killed by cervical dislocation. The hair and skin were saturated with Purasan, and the ventral skin laid back. Three ml of heparinized Hanks' BSS were injected into the peritoneal cavity. The abdomen was massaged, an incision made in the peritoneal wall, and the cell containing fluid removed with a capillary pipette. The harvest from several animals was pooled. A cell count was taken and the fluid adjusted to contain 3×10^6 cells per ml. Cells were planted one ml per flying coverslip tube, placed at 37° C, and allowed to settle for one hour. After settling the medium was decanted and replaced with Eagle's MEM plus combiotic and 30% bovine serum.

Infection of Macrophages

After attachment of cells to coverslips followed by one wash with Hanks' BSS, one ml of virus inoculum was added per coverslip tube. The virus inoculum was made up in Eagle's MEM with or without serum. A zero hour sample of virus inoculum was retained for virus titration. Zero hour was the time virus was added to macrophages.

After a two hour adsorption period at 37° C, the infection medium was decanted, the cells were washed twice with Hanks' BSS to remove residual virus, complete medium was added, and tubes were returned to the incubator.

Coverslips were pulled at two hour adsorption and at 24 hour intervals through 120 hours for staining. Tubes were pulled for virus titration at the same time periods. After three cycles of freeze thaw, the tubes were centrifuged at 3500 rpm to remove cellular debris. The supernatant was decanted, and the virus sample was appropriately diluted and assayed on chicken embryo monolayers in one ounce prescription bottles.

Neutralization Test of Immune Serum

Serum collected from immunized rabbits was heat inactivated at 56° C for 30 minutes. The serum was diluted in Eagle's MEM from 1:5 through 1:1280 using two-fold dilutions. To 1.5 ml of each serum dilution 1.5 ml of virus containing 900 PFU was added. A 1:10 dilution of normal rabbit serum was treated identically. The virus-serum mixtures were incubated in a 37° C waterbath for two hours. Chicken embryo fibroblast monolayers in one ounce prescription bottles were washed with PBS, then inoculated with one ml of the incubated virus-serum mixture. Duplicate bottles were done for each dilution. Adsorption was allowed to occur for two hours, then the virus-serum mixture was removed and Eagle's MEM plus 2% bovine serum and combiotic added. Monolayers were incubated at 37° C for 36 to 48 hours for plaque formation and then stained. The 50% neutralization activity of the serum was determined.

Glassware

Glassware was immersed in a dilute solution of Haemo-sol cleaning solution (Scientific Products, Evanston, Ill.), washed tissue culture clean, rinsed eight to ten times in tap water, and final rinsed in glass distilled deionized water.

Pipettes were soaked in a strong Clorox solution for a minimum of 24 hours, placed in a pipette washer with Alcotab detergent and washed and rinsed for three to four hours. After being rinsed individually in distilled deionized water the pipettes were air dried.

Flying coverslips were soaked for 24 hours in a solution of Haemo-sol, rinsed in running tap water for 24 hours, then rinsed individually ten times in five consecutive distilled deionized water baths and one 95% ethanol bath. The coverslips were stored in 95% ethanol until autoclaved and used.

Staining of Cells on Coverslips

Coverslips were fixed in absolute methanol (5 minutes), stained with May Grunwald (9 minutes) and Giemsa (14 minutes), dehydrated in acetone, cleared in xylene, and mounted on microscope slides with Permount mounting medium.

Concanavalin A Studies

Mice were injected intraperitoneally with 0.5 ml containing 50 ug Concanavalin A (Con A) in Eagle's MEM four days prior to harvest of peritoneal macrophages.

Inhibition of Phagocytosis

(1) Sodium fluoride (NaF)

After macrophages had settled and attached, complete medium containing a concentration of NaF was added. The cells were incubated for two hours, then the medium was removed and replaced with medium containing NaF and a 1:100 dilution of 20% T Staphylococcus epidermidis. Cultures were incubated two hours, stained with May Grunwald Giemsa stain, and examined for evidence of phagocytosis.

(2) 4° C, bacteria

Macrophages, after settling and attaching to coverslips, were placed at 4° C for climatization. When the temperature of the medium stabilized at 4° C it was decanted and replaced with complete medium at 4° C containing Staphylococcus epidermidis as described above to check for phagocytosis.

(3) 4° C, virus

After climatization of macrophages, as above, virus in Eagle's MEM plus 10% bovine serum and combiotic at 4° C was added to macrophage cultures and adsorption allowed to proceed for two hours at 4° C. After adsorption, macrophages were removed from 4° C, infective medium decanted, washed, Eagle's MEM plus 10% bovine serum and combiotic added, and placed at 37° C for remainder of culture period.

Trypsin Treatment of Macrophages

After the macrophages attached to coverslips, 500 ug/ml of trypsin 1:250 in Hanks' BSS was placed on the cells for 45 minutes at 37° C. The trypsin medium was removed, macrophages washed once and infected with virus.

Treatment of Virus with Immune Serum

Virus was suspended in Eagle's MEM plus 20% immune rabbit serum (50% neutralization 1:1280) at a concentration of 3×10^5 PFU per ml. The virus-serum suspension was incubated in a 37° C waterbath for two hours prior to use as an infective medium for rabbit macrophages.

Treatment of Rabbit Macrophages with Immune Serum

Eagle's MEM plus 20% immune rabbit serum was added to settled rabbit macrophages on coverslips. This medium was allowed to remain on the cells for two hours, removed, and the macrophages infected with 3×10^5 PFU per ml of virus in Eagle's MEM plus 10% bovine serum and combiotic.

Electron Microscope Techniques

Immune and nonimmune macrophages attached to coverslips were infected at 6×10^7 PFU/ml and at 3×10^5 PFU/ml in Eagle's MEM plus 10% bovine serum and combiotic. At two hours the cells infected at 6×10^7 were processed for electron microscopy. The remaining infected cells were washed, fresh medium added, and cells processed for electron microscopy at 24, 48, and 72 hours post infection.

For fixation of the cells, the medium was removed and the cells were washed twice with cold 0.1 M phosphate buffer pH 7.4. Cold 4% phosphate buffered gluteraldehyde pH 7.4 was added to the cells for one hour at 4° C, the cells were washed five times with cold buffer and post fixed for 30 minutes in 1% phosphate buffered osmium tetroxide in an ice bath.

Cells were washed twice with buffer, then removed from coverslips

with the flattened edge of an applicator stick and washed from the stick with phosphate buffer into a conical centrifuge tube. After centrifugation at 3500 rpm for ten minutes, the buffer was decanted and the tubes containing cell pellets placed in a 45° C waterbath. 0.05 ml of 2% purified agar was added to each cell pellet. The pellet was broken into clumps and drawn into a warmed capillary pipette. After solidifying, the agar containing the cells was blown out of the capillary pipette onto a sheet of white paper. The black cell clumps were cut out with a razor blade and placed in cold 50% ethanol for 30 minutes, then transferred to 70% and 95% ethanol for 30 minutes each. Cells were then passed through two 30 minute changes of absolute ethanol. The dehydration was carried out at 4° C.

The cells were then transferred to two changes of cold propylene oxide for 30 minutes each and brought to room temperature. A 50:50 mixture of Epon 812 Embedding Media and propylene oxide was placed on the cells in loosely covered containers to allow for the overnight evaporation of propylene oxide.

The cells were then placed in fresh complete Epon 812 for two hours before placing in capsules and filling with Epon 812. Curing was accomplished at 45° C for 12 hours, then at 60° C for 24 hours. After removal from the capsules, the blocks were trimmed and sectioned on an LKB Ultratome. The sections were picked up on parlodion coated 200 mesh grids, allowed to air dry, and stained with Reynold's lead citrate for one minute. The Reynold's lead citrate was routinely filtered before use through a 0.01 micron membrane filter in an attempt to remove any precipitates. Immediately after staining the grids were washed in three changes of

distilled water then air dried. The sections were examined and photographed with a Hitachi HS-8 Electron Microscope.

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

RESULTS

I. Vaccine Vaccinia Virus

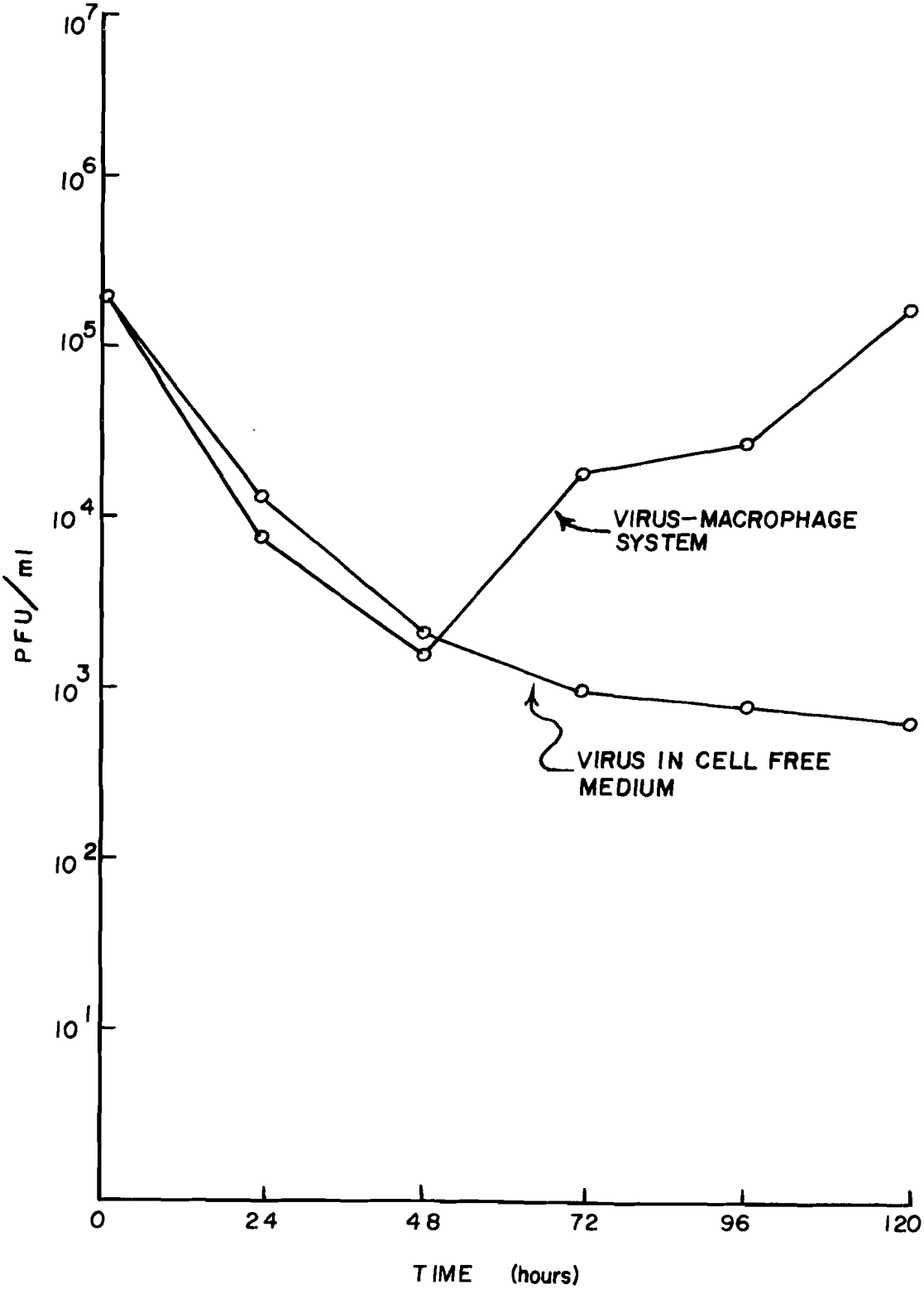
Replication in Rabbit Macrophages

Factors affecting the replication of vaccinia virus in rabbit macrophages were investigated to determine optimum conditions for replication. Macrophages were infected with 3×10^5 PFU/ml of vaccinia virus in complete culture medium at time zero. The infecting medium remained on the macrophages throughout the incubation period. Upon titration of the virus-macrophage system it was found that the infectious viral titer dropped from zero through 48 hours, then increased from 48 through 120 hours (Fig. 1). The viral titer did not rise above the 3×10^5 PFU/ml initially added to the macrophages. An explanation for these observations was sought. Virus was incubated in cell free medium to determine thermal stability of the virus. The incubated virus dropped in titer over the 120 hours due to thermal inactivation (Fig. 1).

Physical limitations inherent in a coverslip situation prevent 100% virus-cell contact. Therefore some of the virus remains free in the medium and does not become cell associated. The thermal inactivation of this free virus from zero through 48 hours accounts for the drop in viral titer as shown in Figure 1.

To eliminate the effect of free virus in the medium on the virus-macrophage system, virus was added for a two hour adsorption period, unadsorbed virus was removed, and fresh medium was added. This procedure gave a clearer indication of the fate of the virus in rabbit

Figure 1: Infection of rabbit peritoneal macrophages with vaccine vaccinia virus and the stability at 37° C of vaccinia in cell-free medium


10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU/ml
VIRUS–MACROPHAGE SYSTEM
VIRUS IN CELL FREE MEDIUM
0
24
48
72
96
120
TIME (hours)

macrophages. The virus adsorbed to and replicated in rabbit macrophages (Fig. 2). After adsorption the titer remained stable from zero through 48 hours. From 48 to 120 hours the viral titer showed an increase indicating that replication occurred in rabbit macrophages. A slight cytopathogenic effect (CPE) was visible in infected cultures (Fig. 3) as compared to uninfected cultures (Fig. 4) at the end of 120 hours of incubation.

The opsonizing effect of serum on the uptake of virus was investigated by allowing adsorption of virus to take place with or without the presence of serum. After adsorption, complete medium containing serum was added to all cultures. There appeared to be a slight increase in the amount of virus adsorbed in the presence of serum (Fig. 5). Replication proceeded similarly with or without the presence of serum during viral adsorption.

A comparison was made as to the effect of the type of serum used in the system. Fetal bovine as compared to bovine serum used in the culture medium had little, if any, effect on the replication of the virus (Fig. 6).

The concentration of serum was also varied to see if this affected the amount or time of replication. Replication occurred equally well in medium supplemented with 10% or 30% bovine serum (Fig. 7).

Replication in Immune Rabbit Macrophages

To determine the effects of the immunological status of an animal on the virus-macrophage interaction, vaccinia virus was added to

Figure 2: Replication of vaccine vaccinia virus in rabbit peritoneal macrophages after two hour viral adsorption

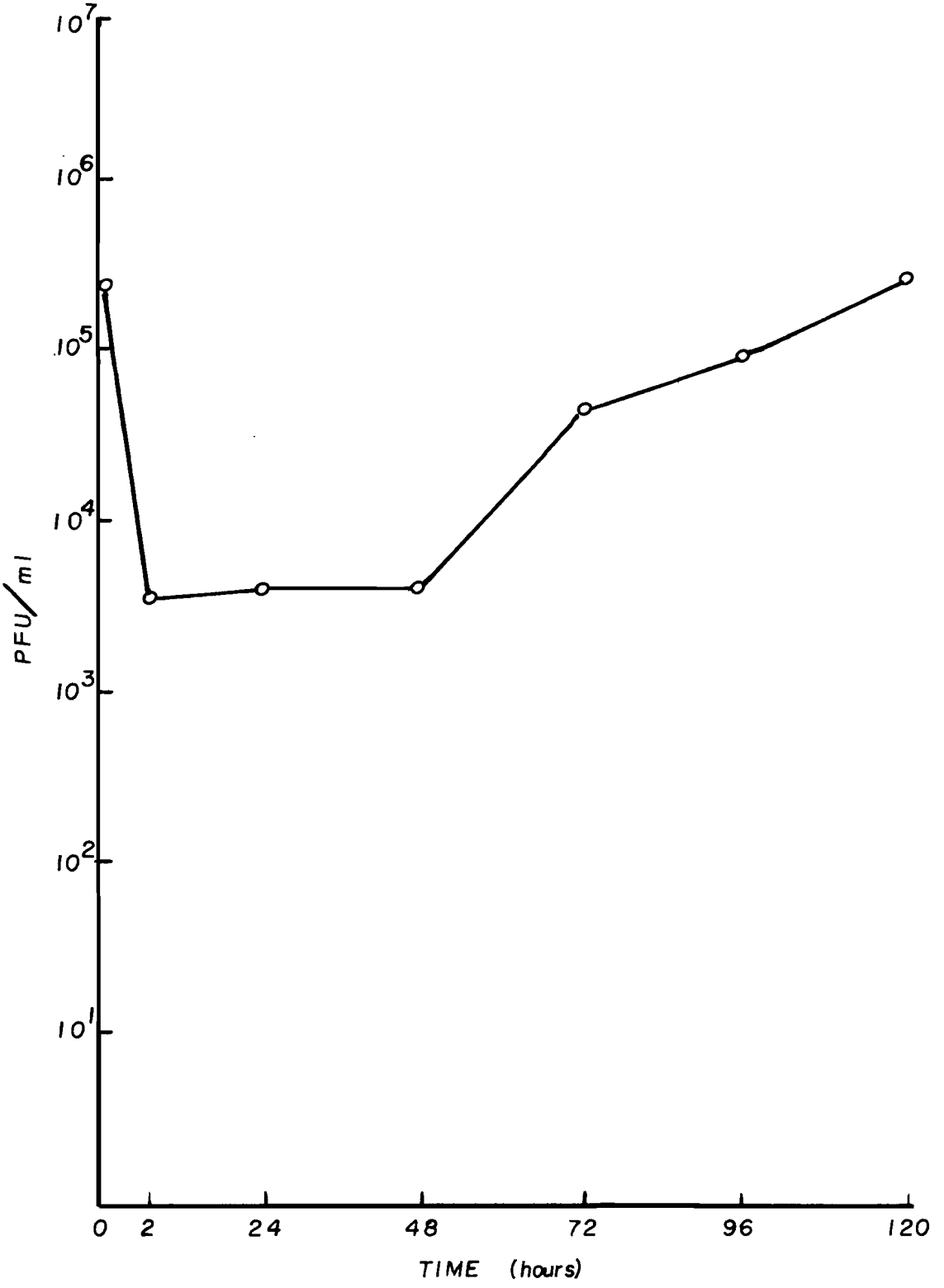
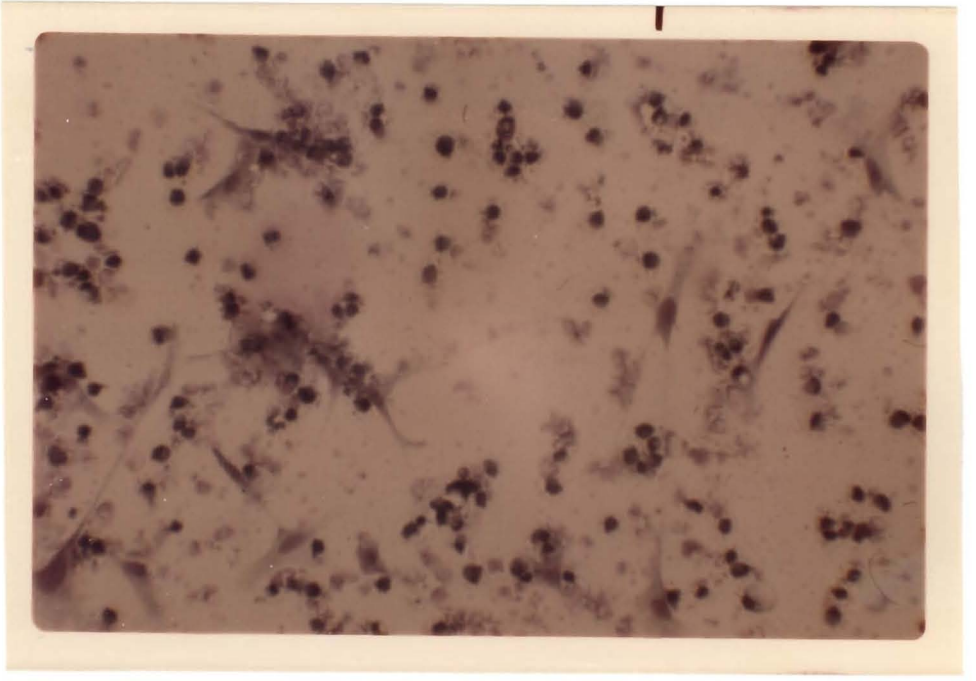

10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU/ml
0
2
24
48
72
96
120
TIME (hours)

Figure 3: Rabbit peritoneal macrophages infected for 120 hours with vaccine vaccinia virus (160X).

Figure 4: Uninfected rabbit peritoneal macrophages at 120 hours (160X).



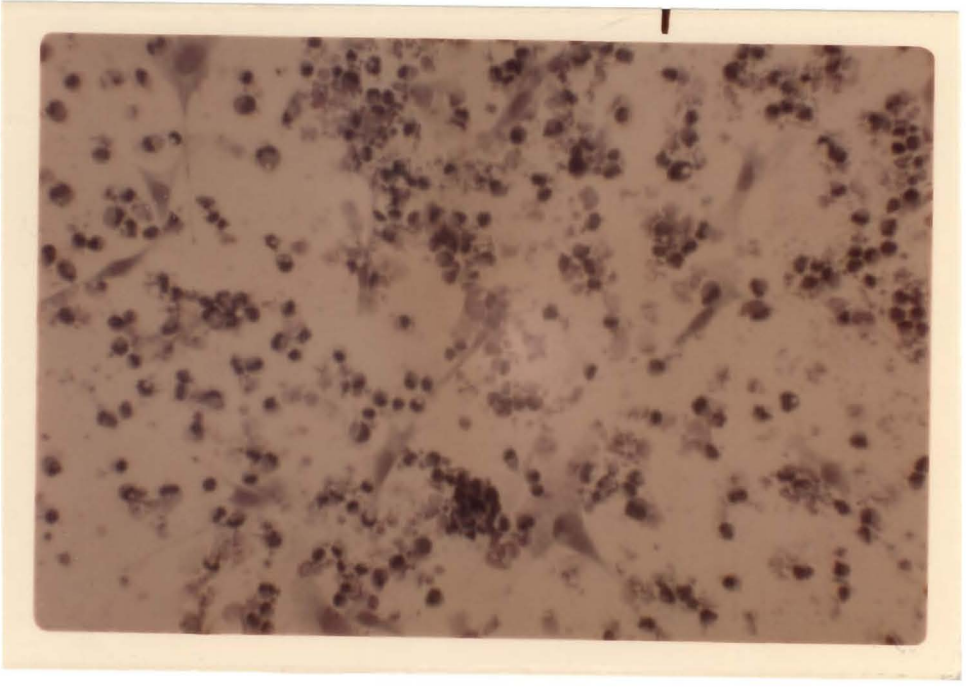


Figure 5: Comparison of replication of vaccine vaccinia virus in rabbit peritoneal macrophages after viral adsorption with or without bovine serum.

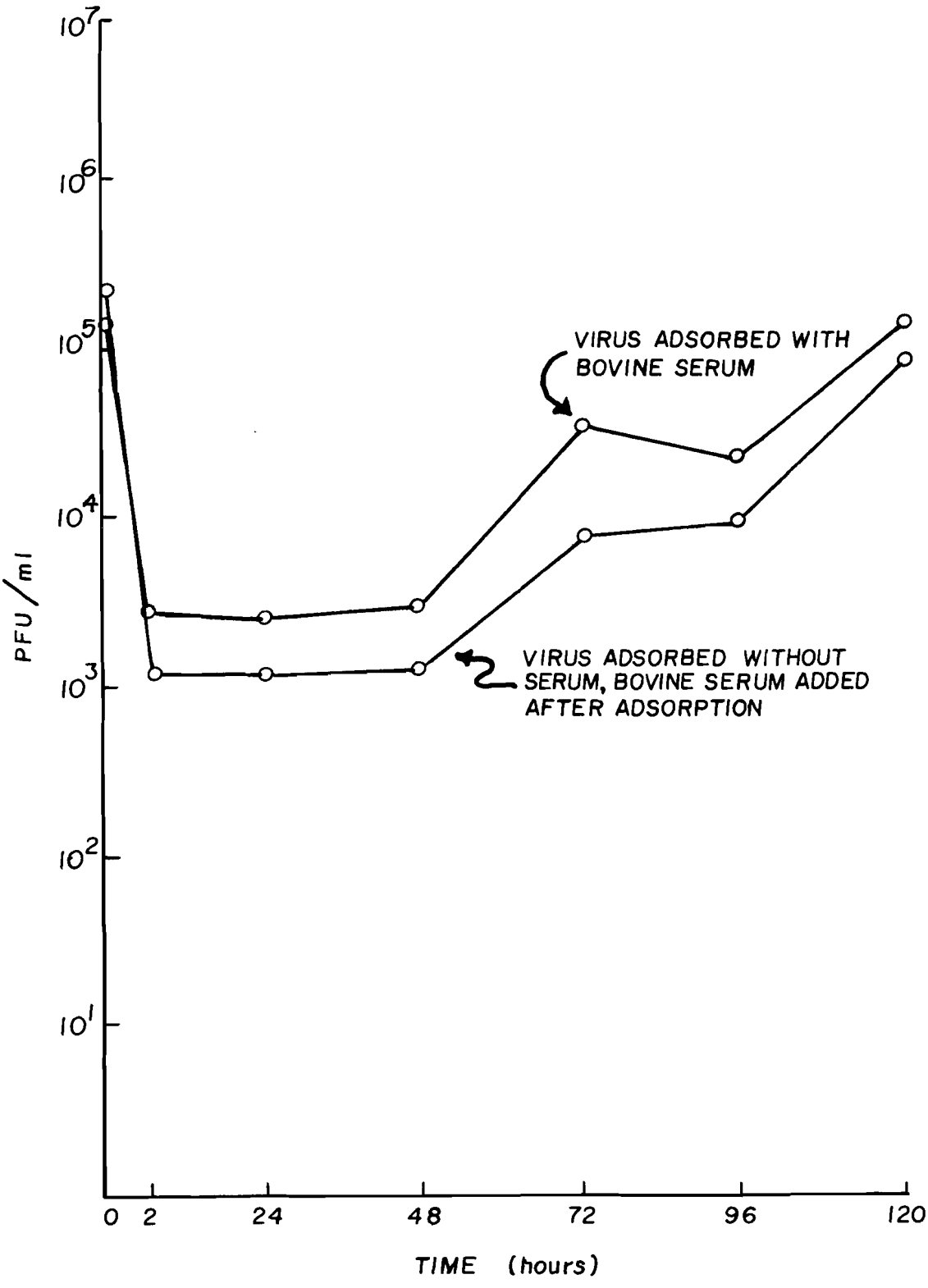

10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU/ml
VIRUS ADSORBED WITH BOVINE SERUM
VIRUS ADSORBED WITHOUT SERUM, BOVINE SERUM ADDED AFTER ADSORPTION
0
2
24
48
72
96
120
TIME (hours)

Figure 6: Comparison of replication of vaccine vaccinia virus in rabbit peritoneal macrophages after viral adsorption with or without fetal bovine serum.

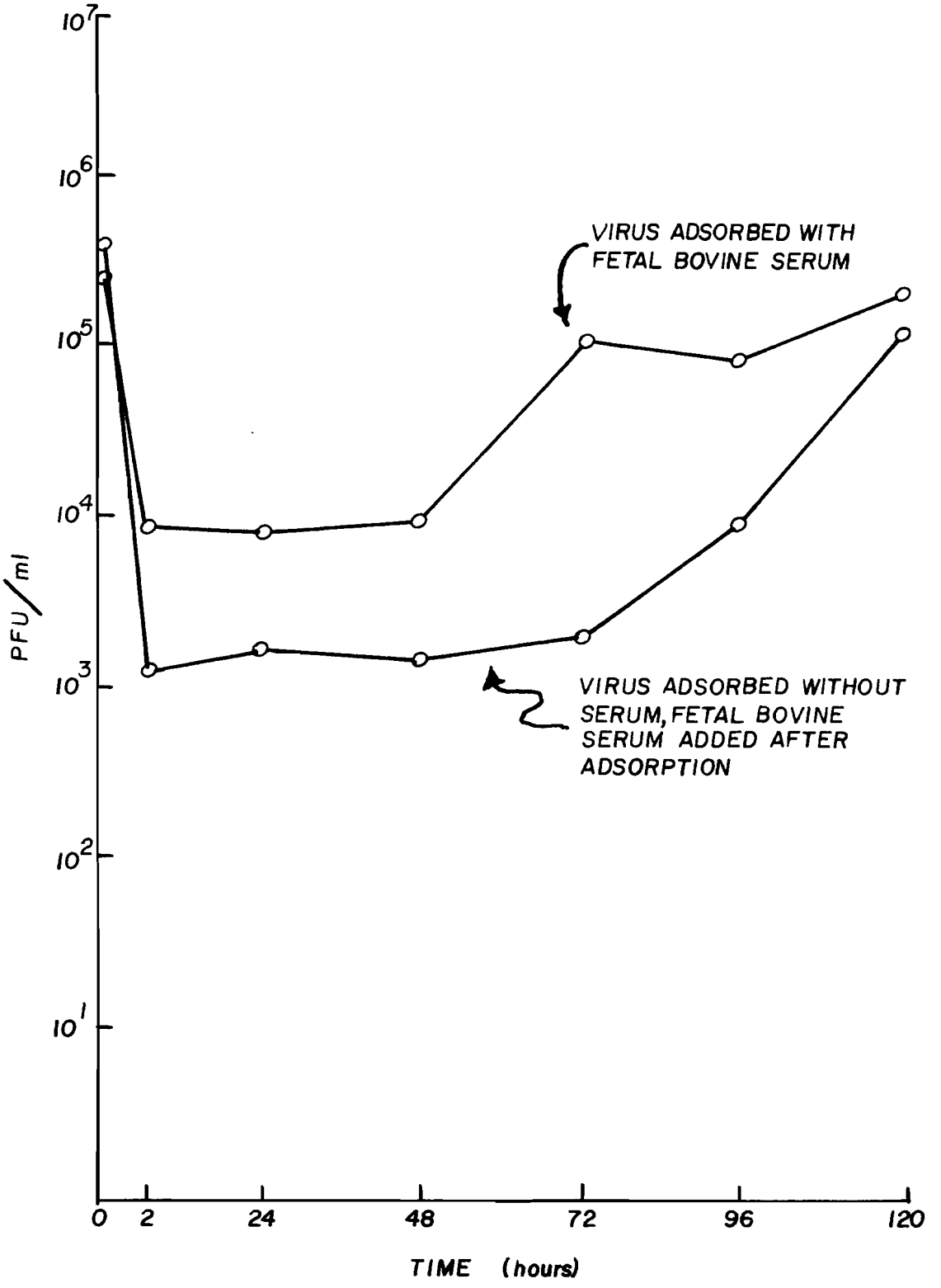
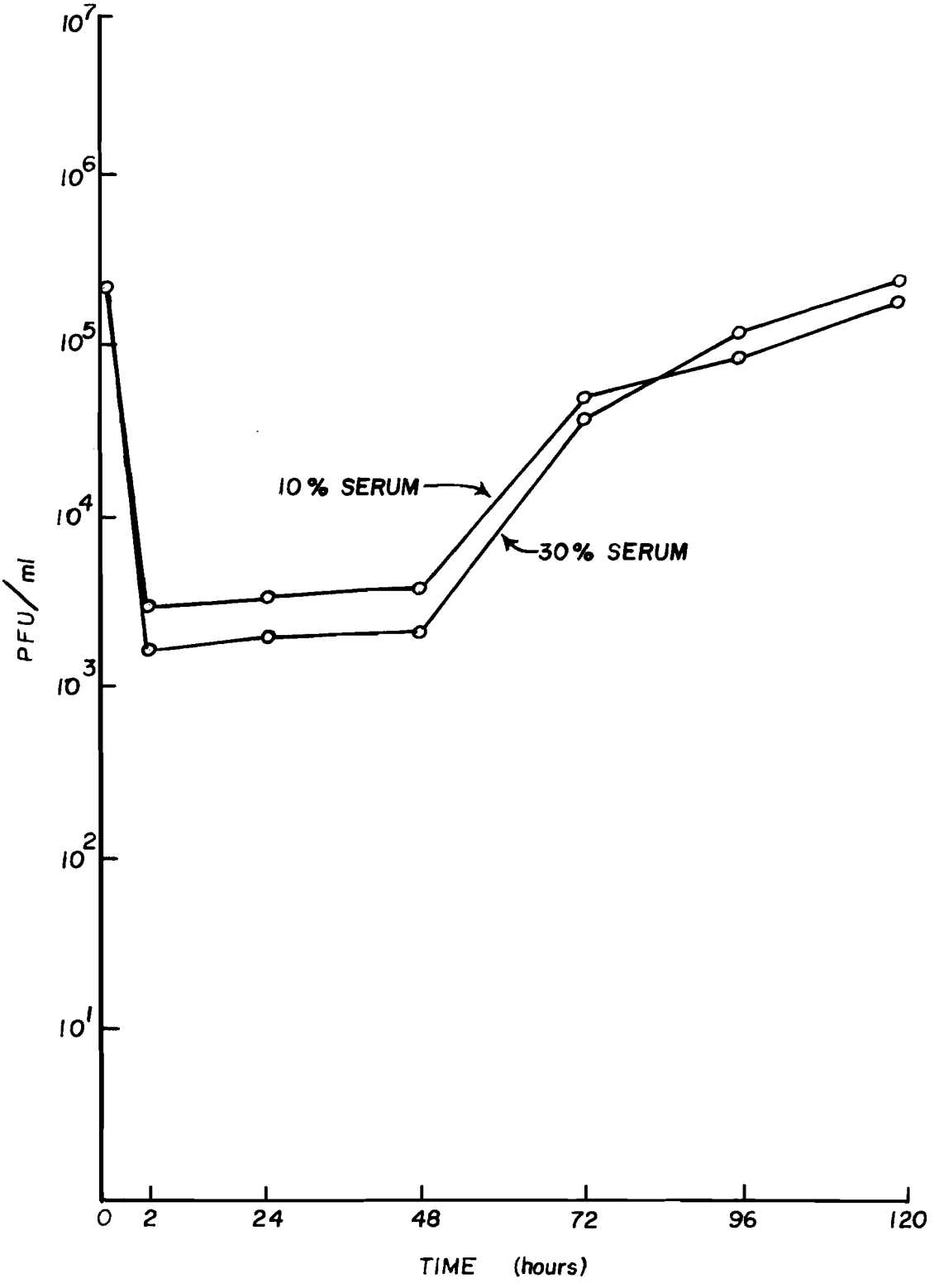

10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU/ml
VIRUS ADSORBED WITH FETAL BOVINE SERUM
VIRUS ADSORBED WITHOUT SERUM, FETAL BOVINE SERUM ADDED AFTER ADSORPTION
0
2
24
48
72
96
120
TIME (hours)

Figure 7: Effect of serum concentration on replication of vaccine vaccinia virus in rabbit peritoneal macrophages.


10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU/ml
10% SERUM
30% SERUM
0
2
24
48
72
96
120
TIME (hours)

macrophages obtained from vaccinia immunized rabbits. Macrophages from immunized animals took up vaccinia virus but failed to support viral replication (Fig. 8). The viral titer dropped slightly with continued incubation of the infected macrophages. There was no visible CPE in infected immune cultures (Fig. 9) observed at 120 hours. The infected cultures appeared to be in the same condition as uninfected immune cultures (Fig. 10).

Immune macrophages were cultured 48 hours before infection to determine the in vitro effect on immune macrophages. There was only a slight difference in the virus interaction as compared to the immediate infection upon being placed in in vitro culture (Fig. 8).

Inhibition of Phagocytosis

Sodium fluoride was used in rabbit macrophage culture medium to ascertain if this chemical could inhibit phagocytosis and thus be used as a tool in investigating viral replication. Three different concentrations (640ug/ml, 320 ug/ml, and 160 ug/ml) of NaF (reported to stop phagocytosis in L cells; Dales and Kajioka, 1964) were added separately to the culture medium of rabbit macrophages. After two hours, killed bacteria in medium containing the appropriate concentration of NaF were added to the cultures to check for phagocytosis. Phagocytosis was observed at all concentrations. Figure 11 is a photograph of cells treated with 640 ug/ml of NaF. There was observed to be a decrease in phagocytosis with increase in concentration of NaF. The heavy cell damage and debris precluded the use of higher concentrations to inhibit

Figure 8: Infection of vaccinia immune rabbit peritoneal macrophages with vaccine vaccinia virus after settling and at 48 hours.

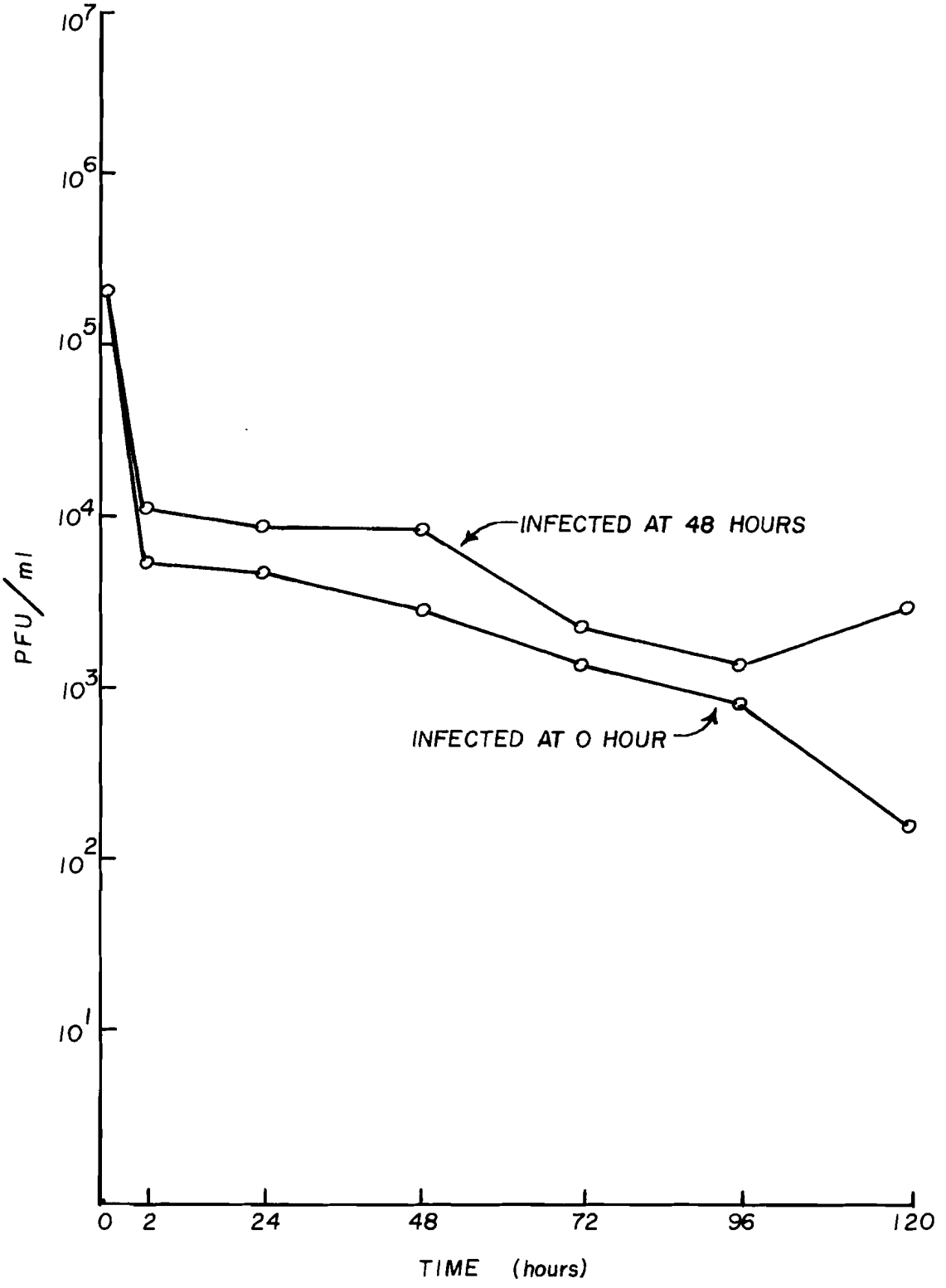

10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU / ml
INFECTED AT 48 HOURS
INFECTED AT 0 HOUR
0
2
24
48
72
96
120
TIME (hours)

Figure 9: Immune rabbit peritoneal macrophages infected with vaccine vaccinia virus for 120 hours. (160X)

Figure 10: Uninfected immune rabbit peritoneal macrophages at 120 hours. (160X)

phagocytosis. Therefore, NaF could not be used to investigate virus-macrophage interaction.

In order to ascertain the effect of cold temperature on the attachment of particles to macrophages and the phagocytosis of these particles by macrophages, the following work was carried out. Rabbit macrophages on coverslips were placed at 4° C until medium temperature stabilized at 4° C (2 hours). Killed bacteria were added to the cultures to check for phagocytosis. At 4° C some bacteria became cell associated, but there was no evidence of ingestion (Fig. 12). In contrast, macrophages to which bacteria were added at 37° C phagocytized the bacteria, and intracellular bacteria were visible (Fig. 13).

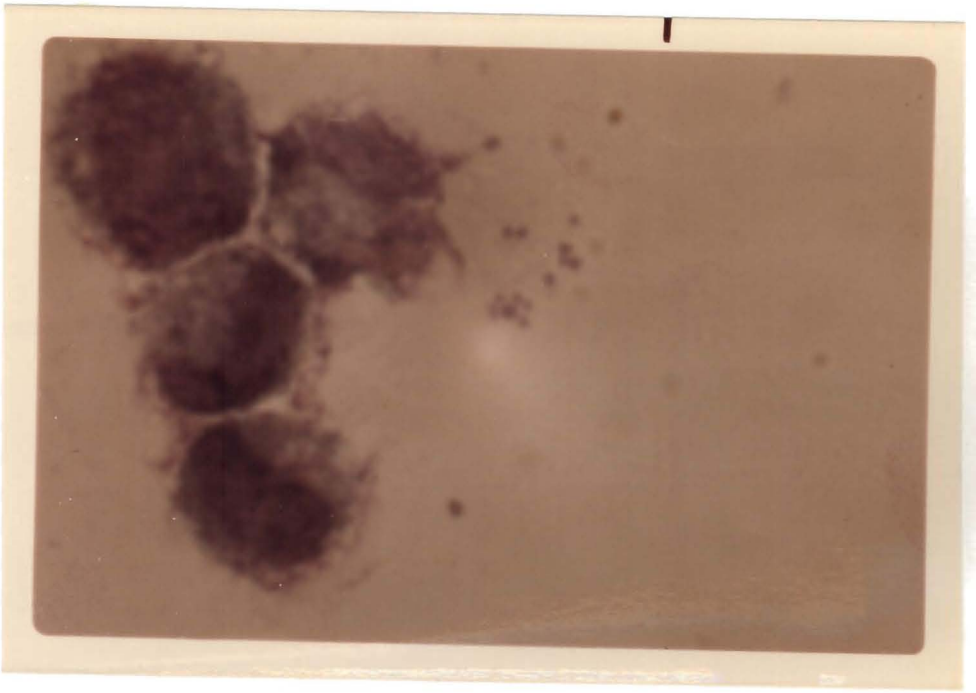
As 4° C inhibited bacterial phagocytosis, macrophages climatized to 4° C were infected with vaccinia virus at 4° C. After two hours the virus medium was removed, the cells were washed, and fresh medium was added. The virus attached to the cells at 4° C as normal replication occurred after incubation at 37° C.

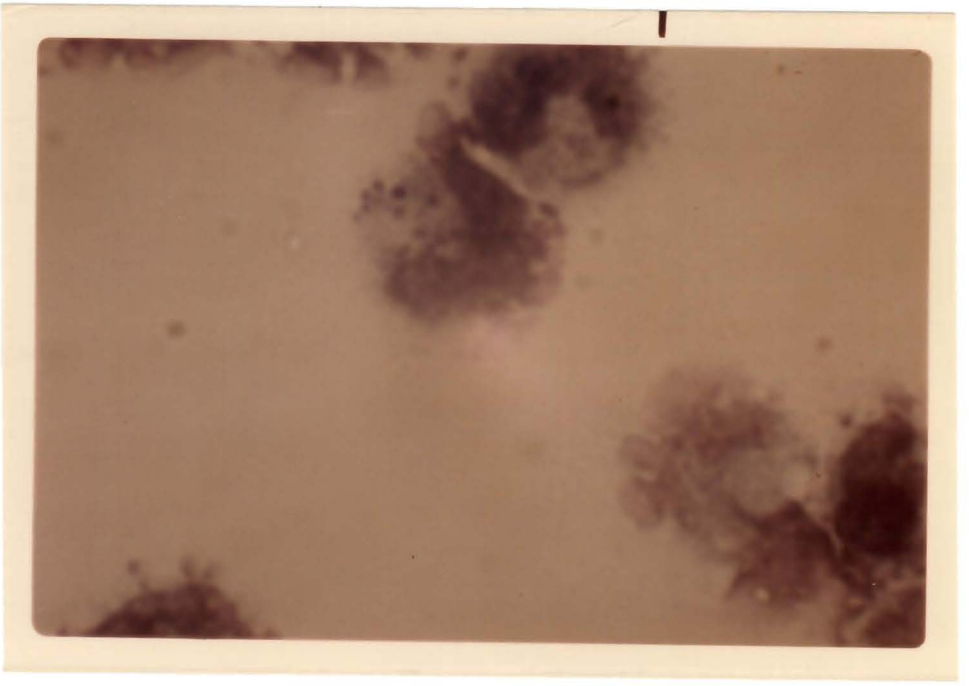
Replication in "Transformed" Cultures

According to Dumont and Sheldon (1965), macrophages undergo nuclear and cytoplasmic changes and can transform into epithelioid cells in both *in vivo* and *in vitro* conditions. After prolonged *in vitro* incubation (168 hours) of rabbit macrophages, the predominating cell type was of epithelial morphology. These cells were found on the entire surface of the coverslip (Fig 14). "Transformed" macrophage cultures were infected with virus to see if the transformation had altered macrophage activity

Figure 12: Phagocytosis of killed S. epidermidis at 4° C after two hours in rabbit peritoneal macrophages. (1600X)

Figure 13: Phagocytosis of killed S. epidermidis at 37° C after two hours in rabbit peritoneal macrophages. (1600X)





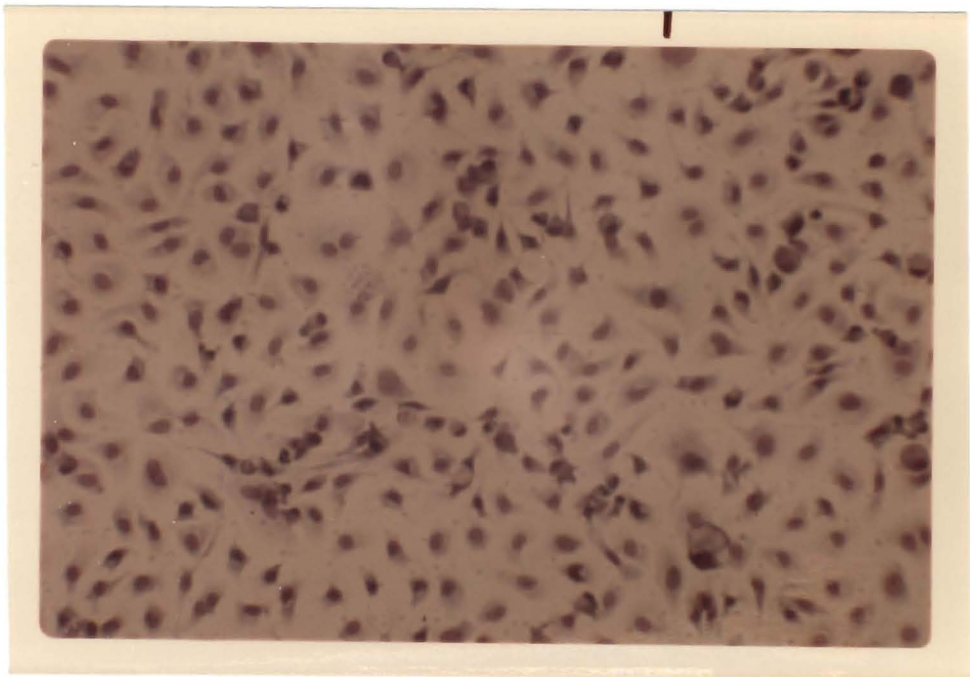


Figure 14: "Transformed" rabbit peritoneal macrophage cultures at 168 hours of incubation. (160X)

with regard to viral handling. When these epithelioid cells, which were derived from normal rabbit macrophage cultures, were infected with vaccinia virus, the virus replicated earlier and with higher titers as compared to results obtained from macrophage cultures which were infected immediately after harvest (Fig. 15). Viral damage to these "transformed" cells was more pronounced (Fig. 16) than the viral damage that occurred in freshly harvested macrophages. This is evidenced by earlier viral injury and CPE and the ultimate production of more virus in the "transformed" cells.

Immune rabbit macrophages also gave rise to "transformed" epithelioid cells. The immune capacity of the "transformed" immune cells was investigated. Vaccinia virus replicated in "transformed" immune cells accompanied by massive cellular destruction (Fig. 17). The amount and pattern of replication did not differ from that observed in normal "transformed" cultures.

II. IHD Vaccinia Virus

Replication in Rabbit Macrophages

The IHD strain of vaccinia virus was used to infect rabbit peritoneal macrophages in the same manner as the vaccine strain to investigate and compare the replication of the two strains in macrophages. The amount of virus adsorbed by macrophages was very similar in the two strains (Fig. 18). However, the subsequent pattern of replication of the two strains differed. The IHD strain increased in titer at each sampling point following adsorption, whereas the vaccine strain increased in titer

Figure 15: Replication of vaccine vaccinia virus in "transformed" rabbit peritoneal macrophage cultures.

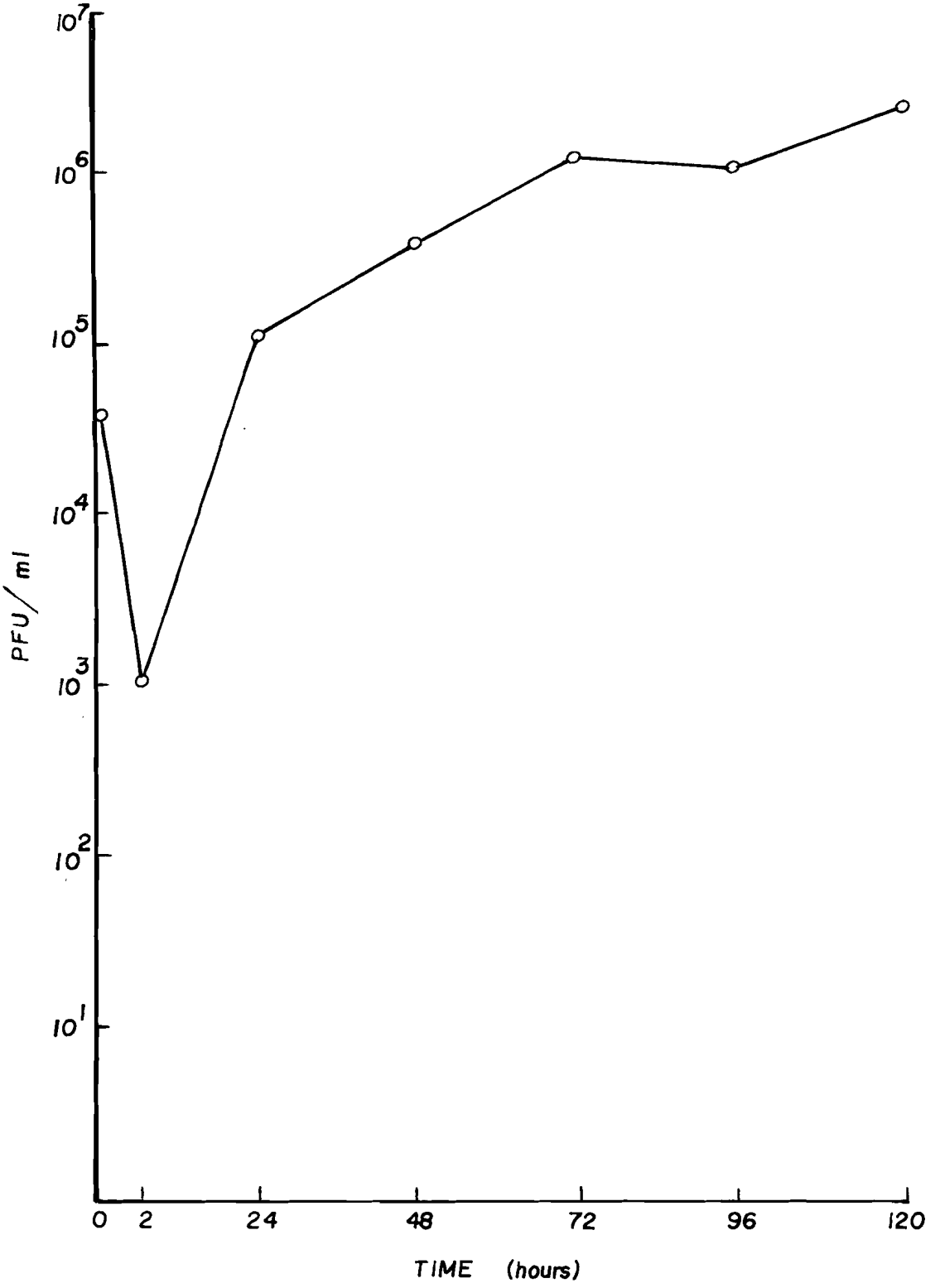
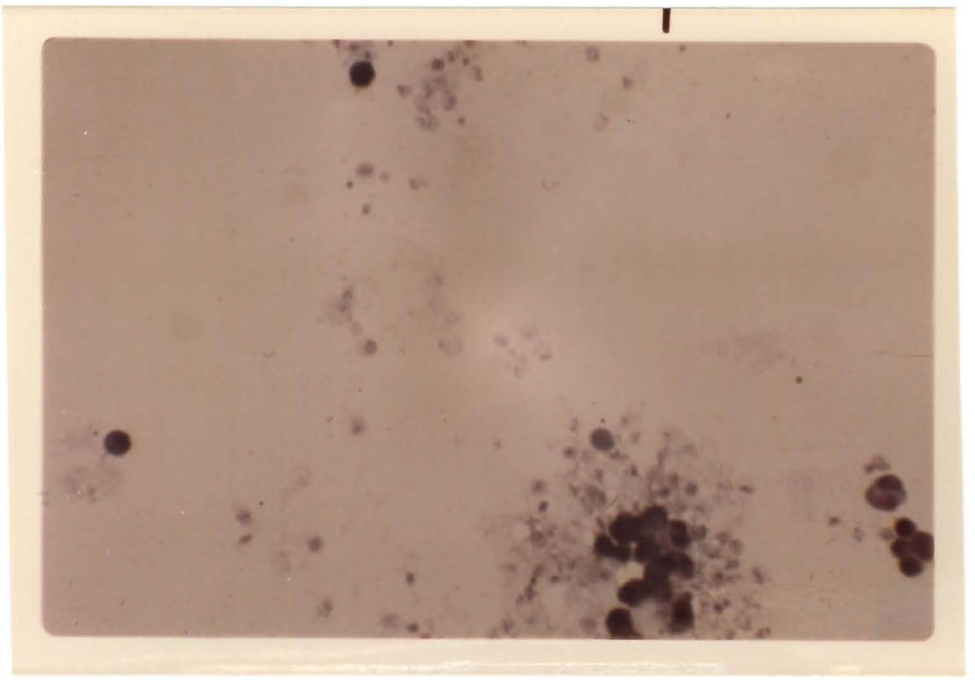

10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU / ml
0
2
24
48
72
96
120
TIME (hours)

Figure 16: Normal "transformed" rabbit peritoneal macrophage cultures infected for 72 hours with vaccine vaccinia virus. (160X)

Figure 17: Immune "transformed" rabbit peritoneal macrophage cultures infected for 72 hours with vaccine vaccinia virus. (160X)



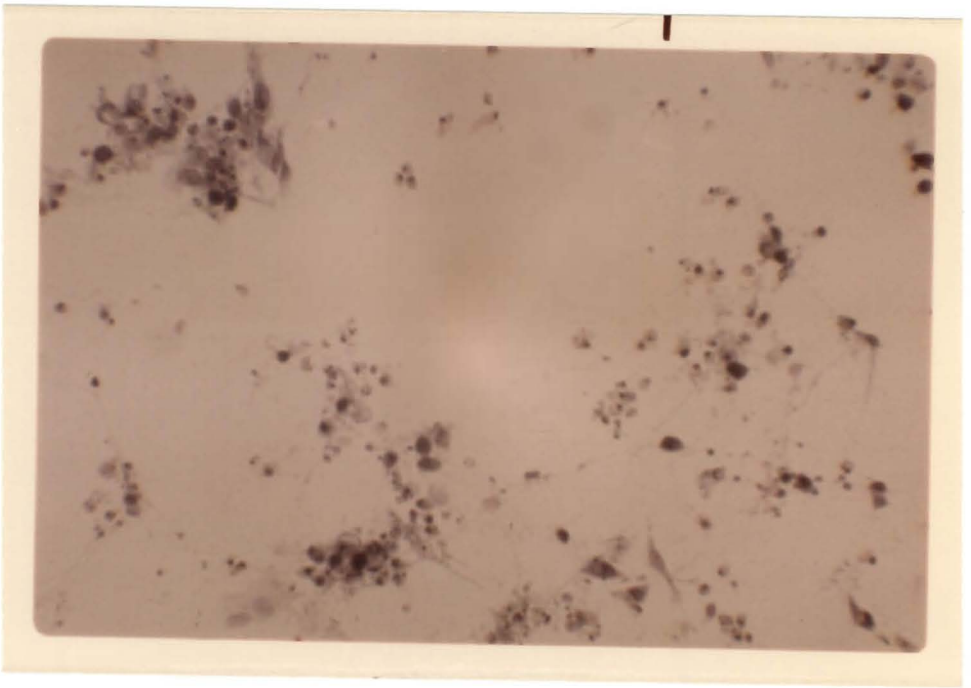
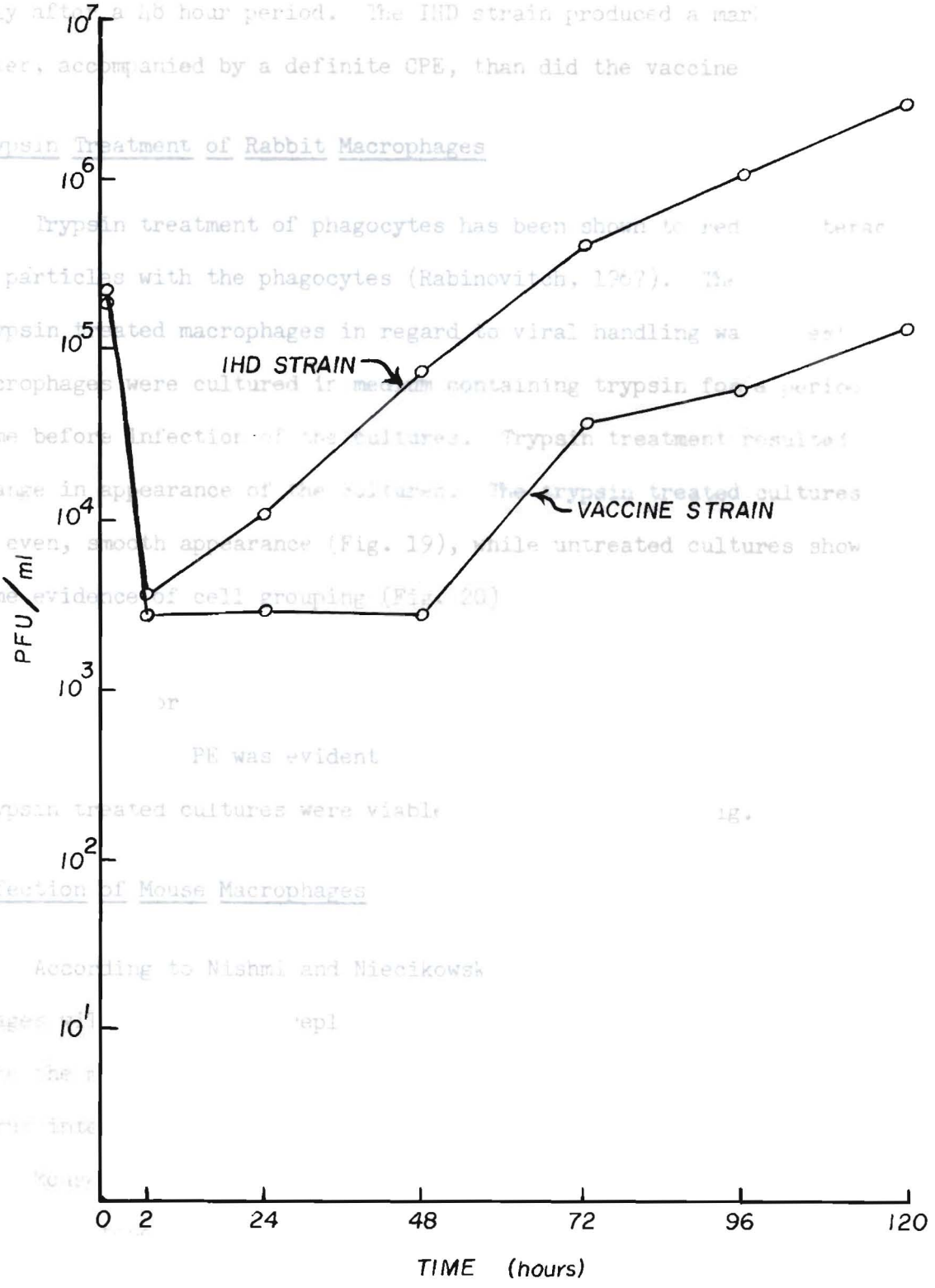


Figure 18: Comparison of replication of vaccine and IHD strains of vaccinia virus in rabbit peritoneal macrophages.


10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU / ml
IHD STRAIN
VACCINE STRAIN
0
2
24
48
72
96
120
TIME (hours)

only after a 48 hour period. The IHD strain produced a markedly higher titer, accompanied by a definite CPE, than did the vaccine strain.

Trypsin Treatment of Rabbit Macrophages

Trypsin treatment of phagocytes has been shown to reduce interaction of particles with the phagocytes (Rabinovitch, 1967). The effect of trypsin treated macrophages in regard to viral handling was investigated. Macrophages were cultured in medium containing trypsin for a period of time before infection of the cultures. Trypsin treatment resulted in a change in appearance of the cultures. The trypsin treated cultures had an even, smooth appearance (Fig. 19), while untreated cultures showed some evidence of cell grouping (Fig. 20).

The trypsin treatment of the macrophages did not prevent infection of the cells nor replication of the virus (Fig. 21). At 72 hours after infection viral CPE was evident (Fig. 22), while 72 hour uninfected trypsin treated cultures were viable, stable cultures (Fig. 23).

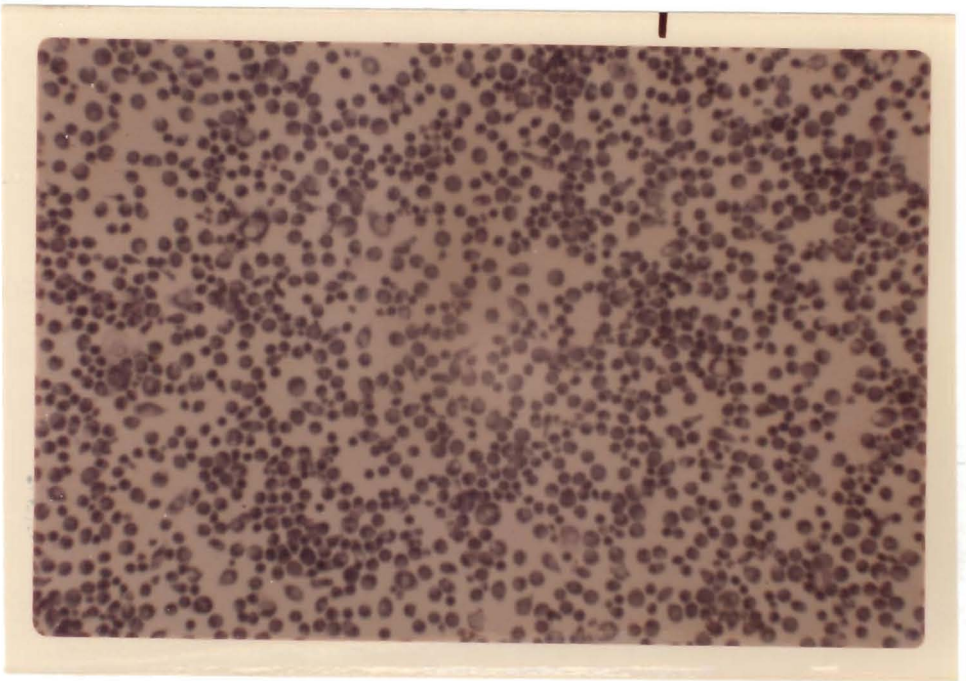
Infection of Mouse Macrophages

According to Nishmi and Niecikowski (1963), mouse peritoneal macrophages will not support replication of vaccinia virus. In order to compare the mouse macrophage-virus interaction with the rabbit macrophage-virus interaction, the following experiments were performed.

Mouse macrophages were infected with 3×10^5 PFU/ml of IHD virus. Mouse peritoneal macrophages infected with vaccinia virus did not support replication of the virus (Fig. 24). The viral titer gradually

Figure 19: Rabbit peritoneal macrophages treated with 500 ug/ml of trypsin for 45 minutes at 37° C. (160X)

Figure 20: Untreated rabbit peritoneal macrophages after 45 minutes in Eagle's MEM culture medium. (160X)



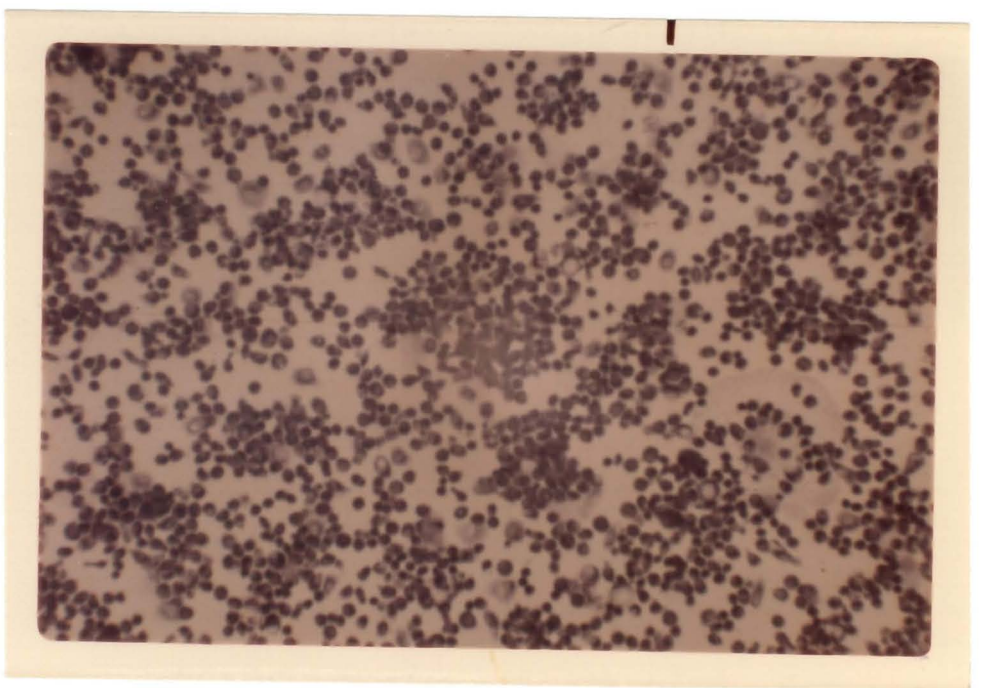


Figure 21: Replication of IHD vaccinia virus in trypsin treated rabbit peritoneal macrophages.

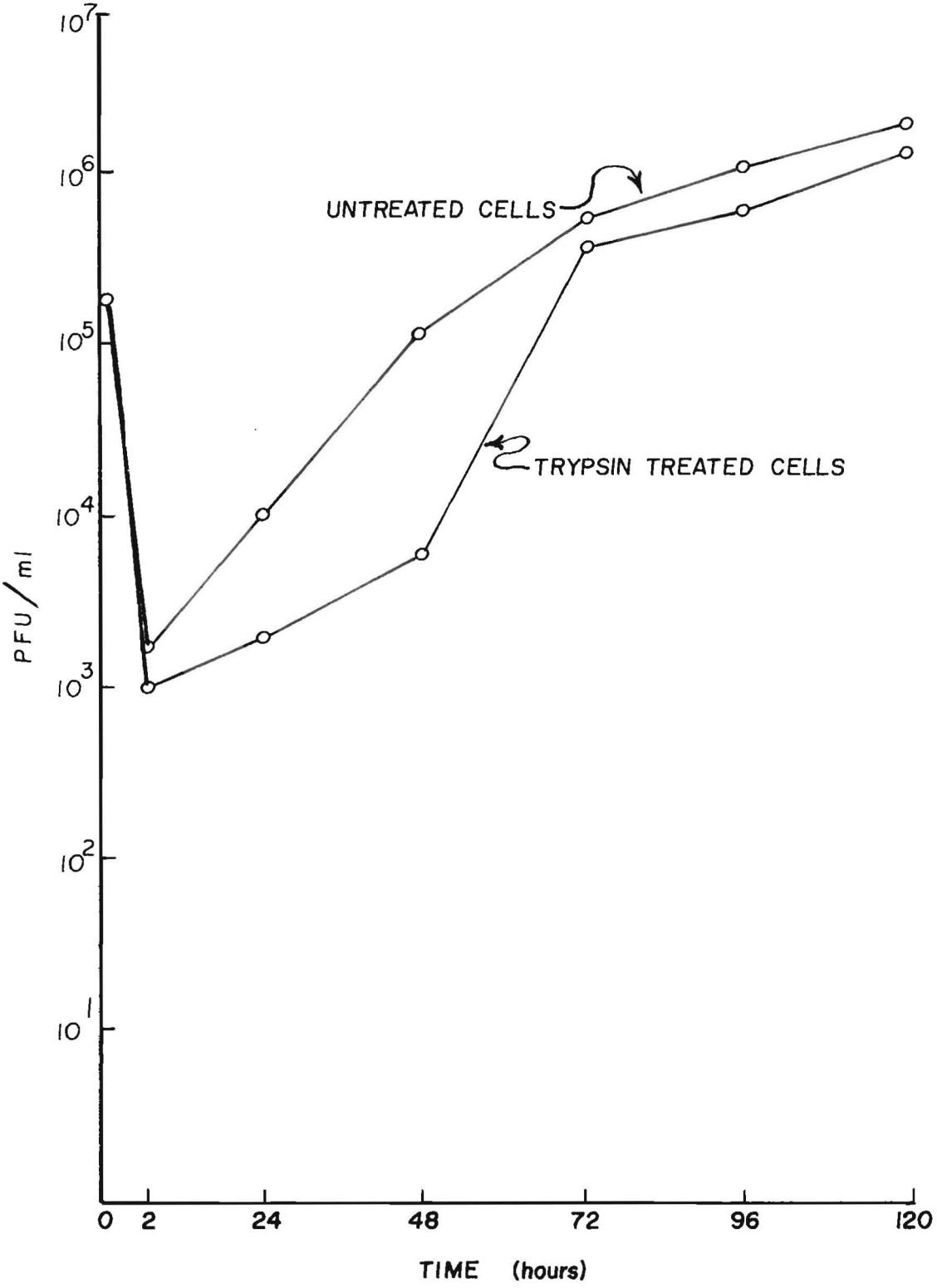
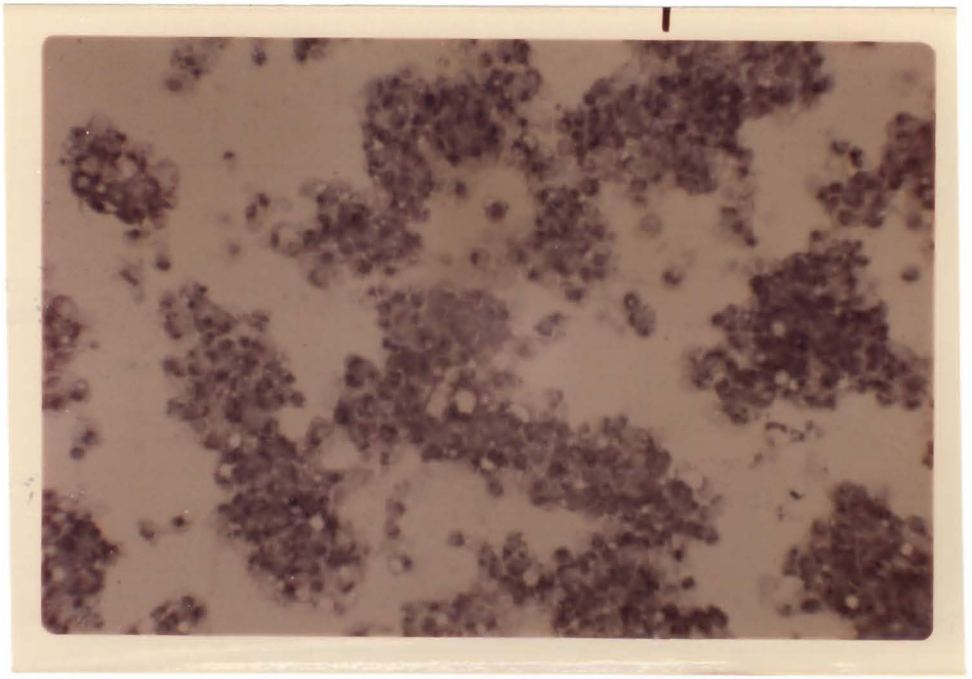

10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU/ml
UNTREATED CELLS
TRYPSIN TREATED CELLS
0
2
24
48
72
96
120
TIME (hours)

Figure 22: Trypsin treated rabbit peritoneal macrophages infected for 72 hours with IHD vaccinia virus. (160X)

Figure 23: Uninfected trypsin treated macrophages at 72 hours. (160X)



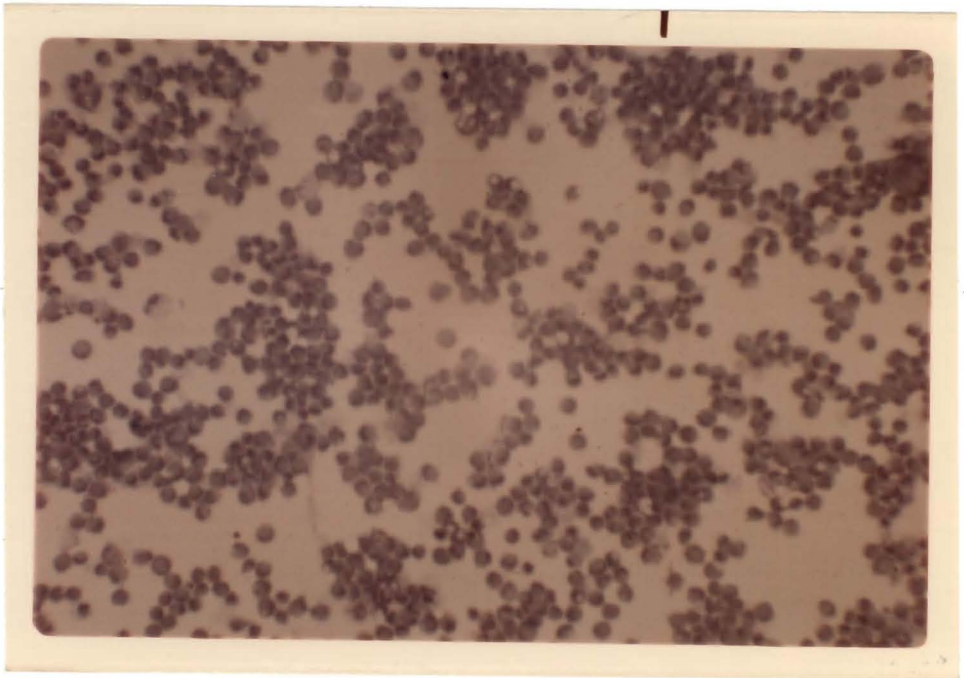
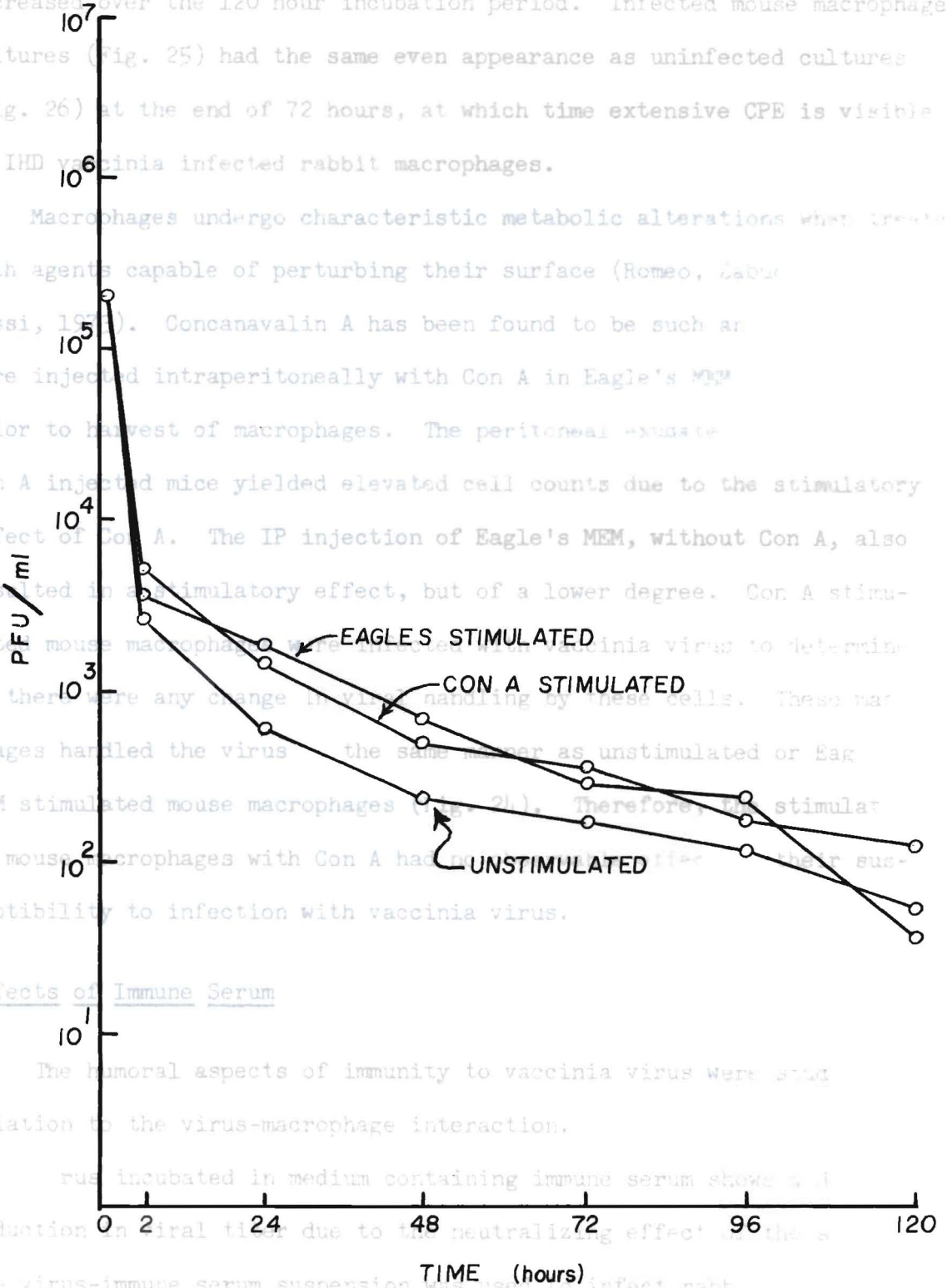


Figure 24: Infection of unstimulated, Con A stimulated, and Eagle's MEM stimulated mouse peritoneal macrophages with IHD vaccinia virus.


10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU / ml
EAGLE'S STIMULATED
CON A STIMULATED
UNSTIMULATED
0
2
24
48
72
96
120
TIME (hours)

decreased over the 120 hour incubation period. Infected mouse macrophage cultures (Fig. 25) had the same even appearance as uninfected cultures (Fig. 26) at the end of 72 hours, at which time extensive CPE is visible in IHD vaccinia infected rabbit macrophages.

Macrophages undergo characteristic metabolic alterations when treated with agents capable of perturbing their surface (Romeo, Zabucchi, and Rossi, 1973). Concanavalin A has been found to be such an agent. Mice were injected intraperitoneally with Con A in Eagle's MEM four days prior to harvest of macrophages. The peritoneal exudates obtained from Con A injected mice yielded elevated cell counts due to the stimulatory effect of Con A. The IP injection of Eagle's MEM, without Con A, also resulted in a stimulatory effect, but of a lower degree. Con A stimulated mouse macrophages were infected with vaccinia virus to determine if there were any change in viral handling by these cells. These macrophages handled the virus in the same manner as unstimulated or Eagle's MEM stimulated mouse macrophages (Fig. 24). Therefore, the stimulation of mouse macrophages with Con A had no observable effect on their susceptibility to infection with vaccinia virus.

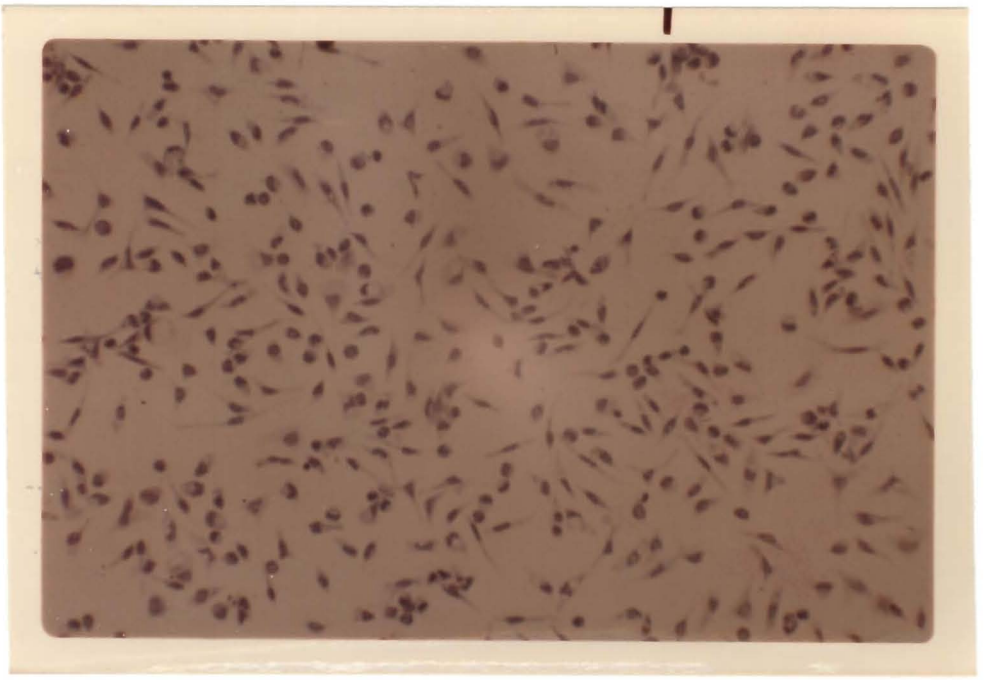
Effects of Immune Serum

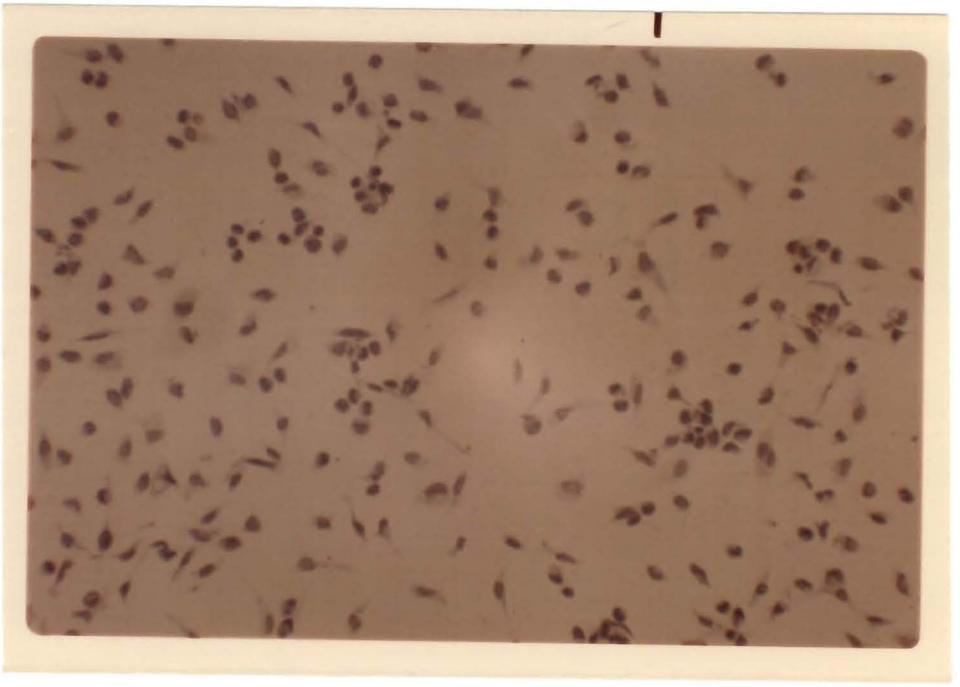
The humoral aspects of immunity to vaccinia virus were studied in relation to the virus-macrophage interaction.

Virus incubated in medium containing immune serum shows a definite reduction in viral titer due to the neutralizing effect of the serum. The virus-immune serum suspension was used to infect rabbit peritoneal macrophages. After adsorption the virus was undetectable due to the

Figure 25: Mouse peritoneal macrophages infected for 72 hours with IHD vaccinia virus. (160X)

Figure 26: Uninfected mouse peritoneal macrophages at 72 hours. (160X)





neutralization. Virus did not replicate in rabbit macrophages after being incubated with immune serum; however, virus incubated with normal rabbit serum replicated in rabbit macrophages indicating that the lack of replication was due to the presence of antibody in the serum (Fig. 27). No CPE was initiated in cultures infected with virus preincubated with immune serum (Fig. 28).

In order to determine if cytophilic antibody was present in vaccinia immune serum, rabbit macrophages were incubated in medium containing immune serum; the immune serum was removed and infecting virus was added. The macrophages treated with immune serum adsorbed nearly the same amount of virus as macrophages treated with normal serum (Fig. 29). The virus replicated in macrophages which had been incubated with either immune or normal rabbit serum. The immune serum, when used to treat macrophages did not prevent the occurrence of viral CPE in infected cultures (Fig. 30).

Comparison of Replication in Immune and Nonimmune Rabbit Macrophages

Viral replication occurred in macrophages obtained from nonimmune rabbits but not in macrophages obtained from vaccinia immune rabbits. Experiments were run to compare the two systems, and the following results were obtained.

The amount of virus adsorbed by immune macrophages was equal to the amount of virus adsorbed by nonimmune macrophages (Fig. 31). The viral titer remained essentially the same in the immune system compared to an increase in viral titer in the nonimmune system. The divergent paths of virus in each cell system were followed by examination of each system at

Figure 27: Infection of rabbit peritoneal macrophages with IHD vaccinia virus incubated two hours with vaccinia immune or normal rabbit serum before viral adsorption.

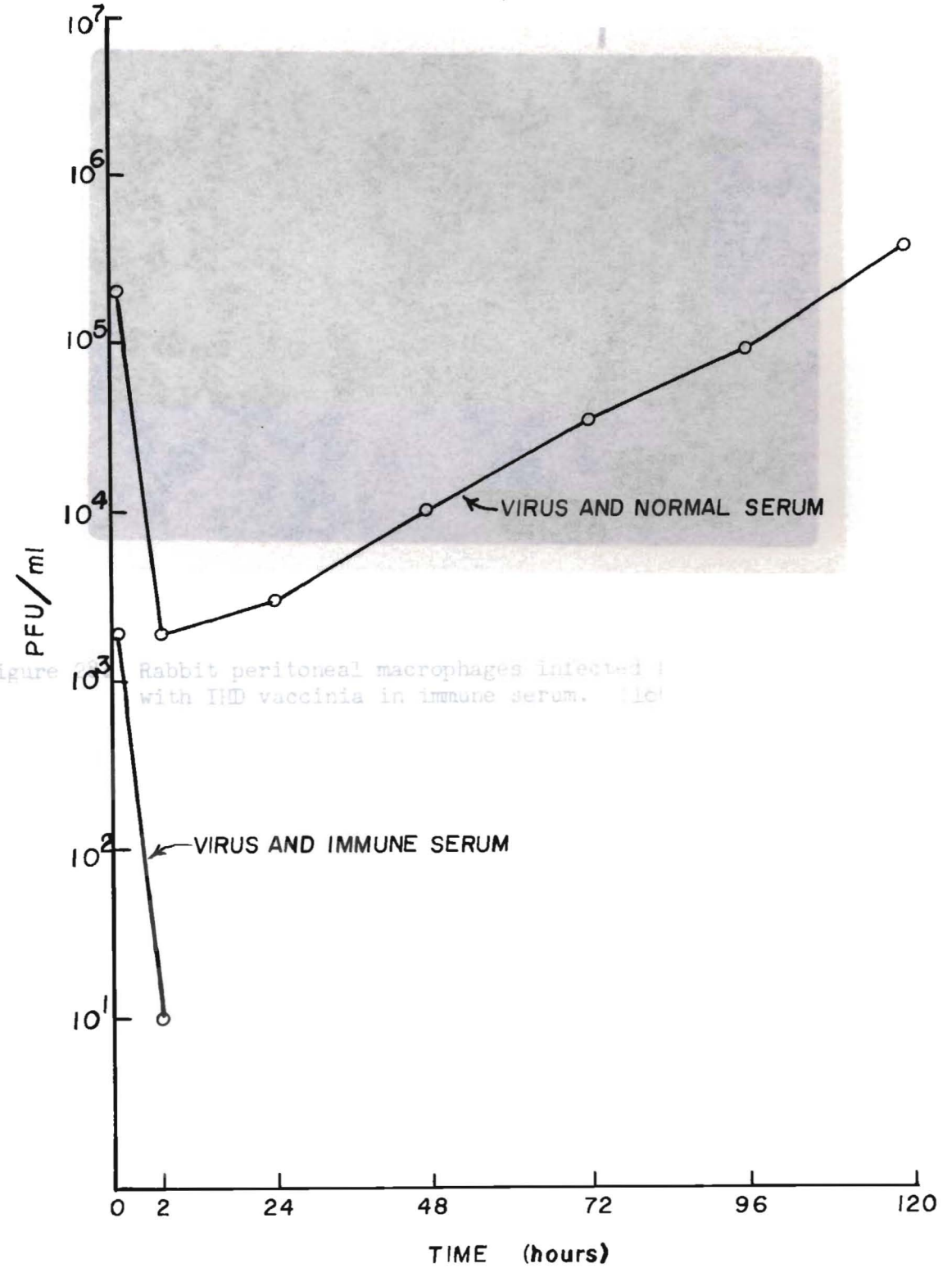


Figure [illegible] Rabbit peritoneal macrophages infected [illegible]
with IHD vaccinia in immune serum. [illegible]

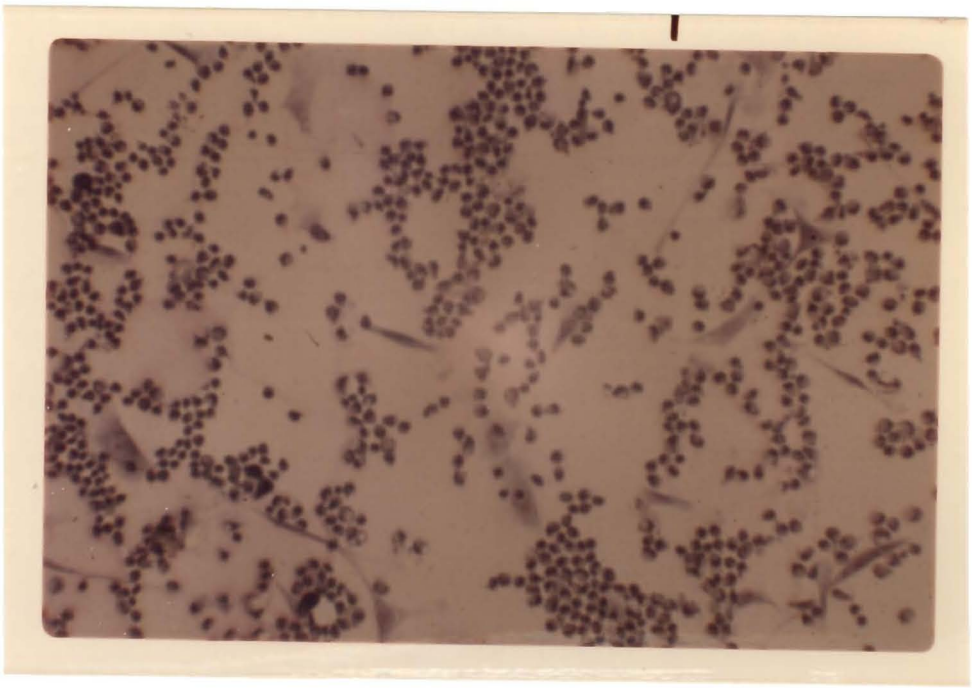
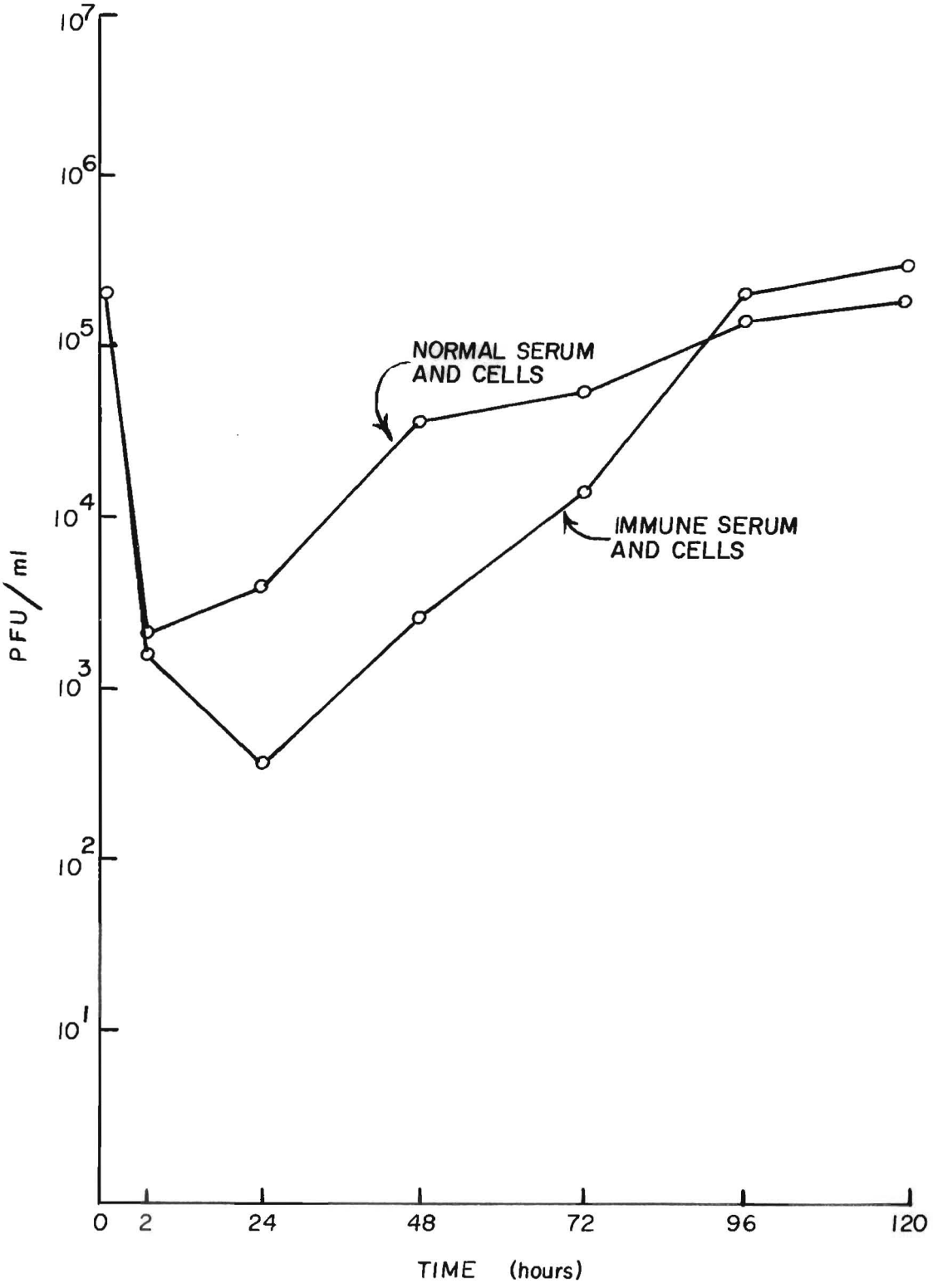


Figure 28: Rabbit peritoneal macrophages infected for 72 hours with IHD vaccinia in immune serum. (160X)

Figure 29: Replication of IHD vaccinia virus in rabbit peritoneal macrophages incubated two hours with vaccinia immune or normal rabbit serum before infection.


10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU / ml
NORMAL SERUM
AND CELLS
IMMUNE SERUM
AND CELLS
0
2
24
48
72
96
120
TIME (hours)

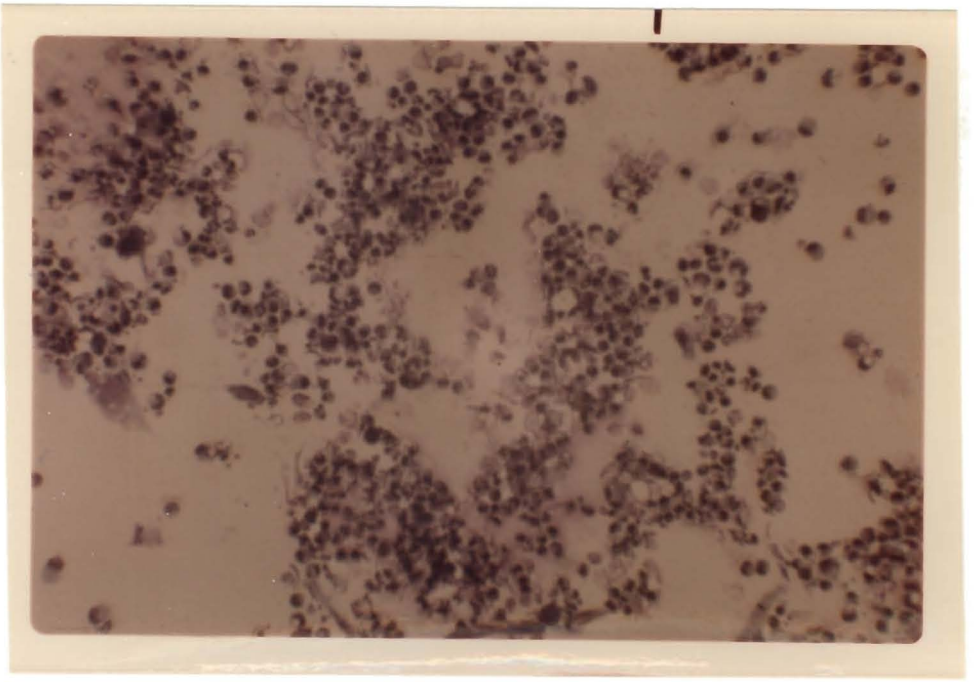
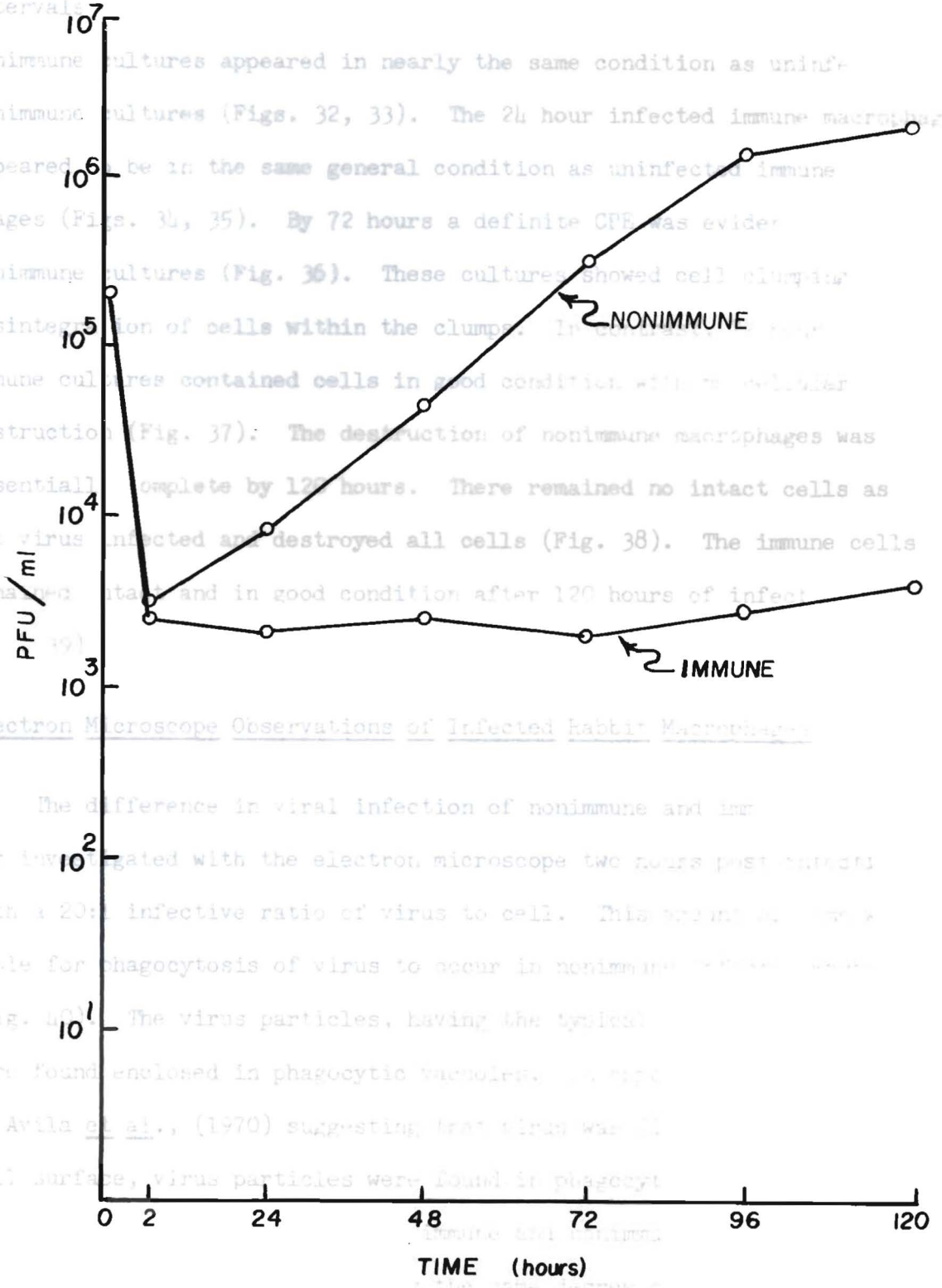


Figure 30: Rabbit peritoneal macrophages incubated with immune serum before infection with IHD vaccinia virus for 72 hours. (160X)

Figure 31: Infection of nonimmune and vaccinia immune rabbit peritoneal macrophages with IHD vaccinia virus.


10^7
10^6
10^5
10^4
10^3
10^2
10^1
PFU/ml
NONIMMUNE
IMMUNE
0
2
24
48
72
96
120
TIME (hours)

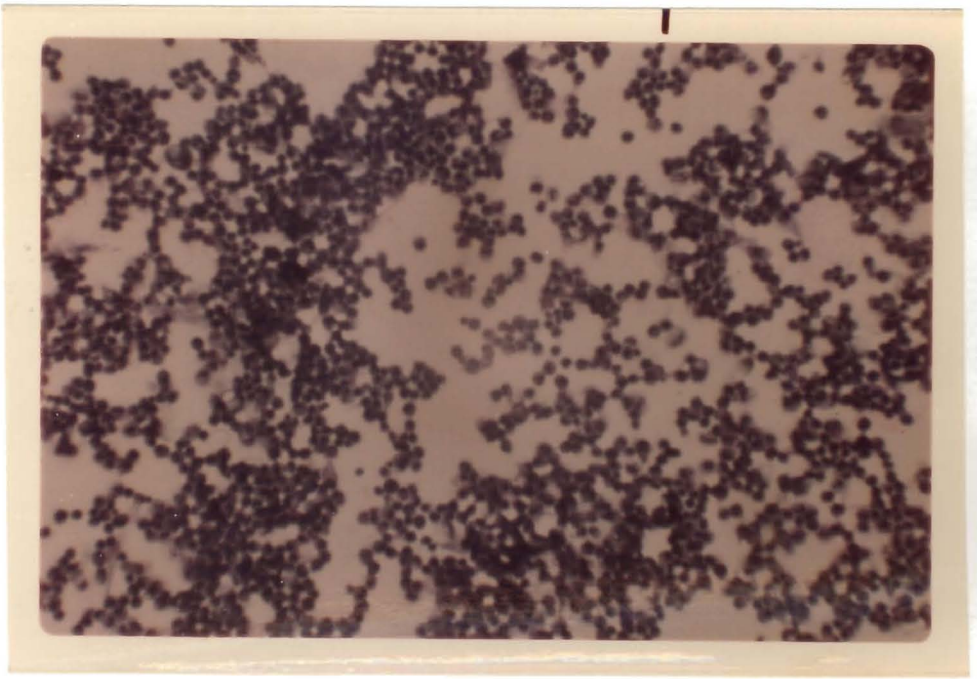
intervals throughout the incubation period. At 24 hours after infection nonimmune cultures appeared in nearly the same condition as uninfected nonimmune cultures (Figs. 32, 33). The 24 hour infected immune macrophages appeared to be in the same general condition as uninfected immune macrophages (Figs. 34, 35). By 72 hours a definite CPE was evident in infected nonimmune cultures (Fig. 36). These cultures showed cell clumping and disintegration of cells within the clumps. In contrast, 72 hour infected immune cultures contained cells in good condition with no cellular destruction (Fig. 37). The destruction of nonimmune macrophages was essentially complete by 120 hours. There remained no intact cells as the virus infected and destroyed all cells (Fig. 38). The immune cells remained intact and in good condition after 120 hours of infection (Fig. 39).

Electron Microscope Observations of Infected Rabbit Macrophages

The difference in viral infection of nonimmune and immune macrophages was investigated with the electron microscope two hours post infection with a 20:1 infective ratio of virus to cell. This amount of time was ample for phagocytosis of virus to occur in nonimmune infected macrophages (Fig. 40). The virus particles, having the typical dumbbell shaped core, were found enclosed in phagocytic vacuoles. In opposition to the theory of Avila et al., (1970) suggesting that virus was fixed at the immune cell surface, virus particles were found in phagocytic vacuoles of the immune cells (Fig. 41). Vaccinia immune and nonimmune rabbit macrophages phagocytized the virus with nearly the same degree of occurrence.

Figure 32: Nonimmune rabbit peritoneal macrophages infected for 24 hours with IHD vaccinia virus. (160X)

Figure 33: Uninfected nonimmune rabbit peritoneal macrophages at 24 hours. (160X)



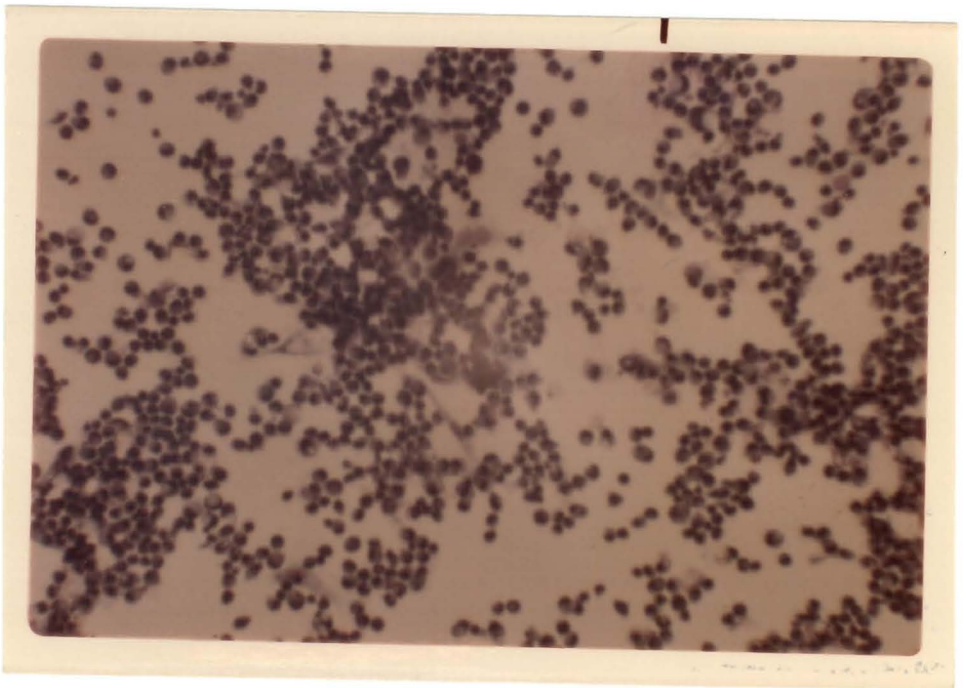
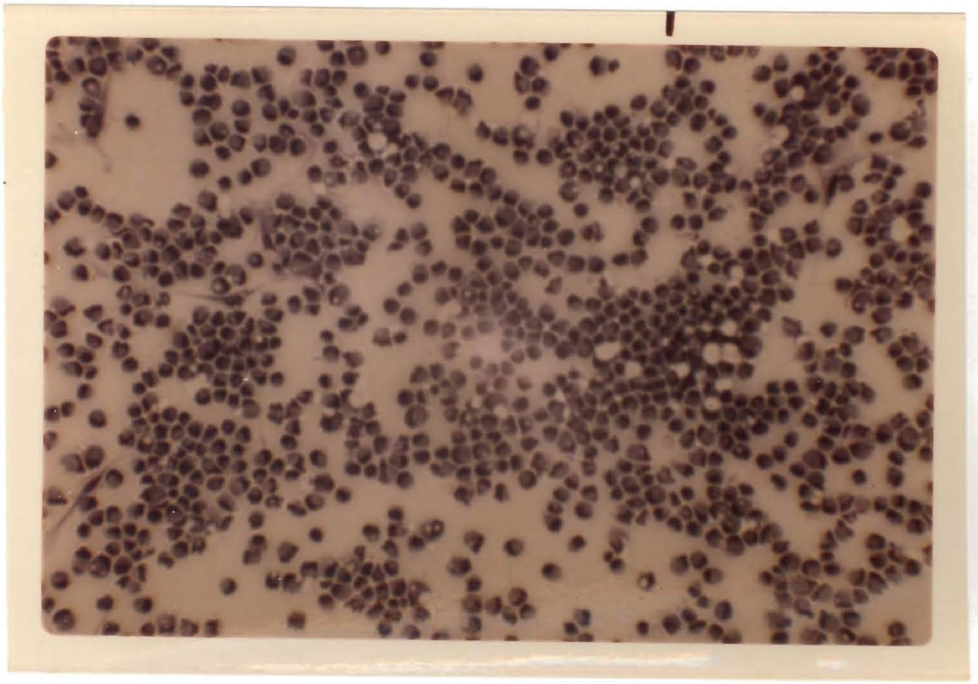


Figure 34: Immune rabbit peritoneal macrophages infected for 24 hours with IHD vaccinia virus. (160X)

Figure 35: Uninfected immune rabbit peritoneal macrophages at 24 hours. (160X)



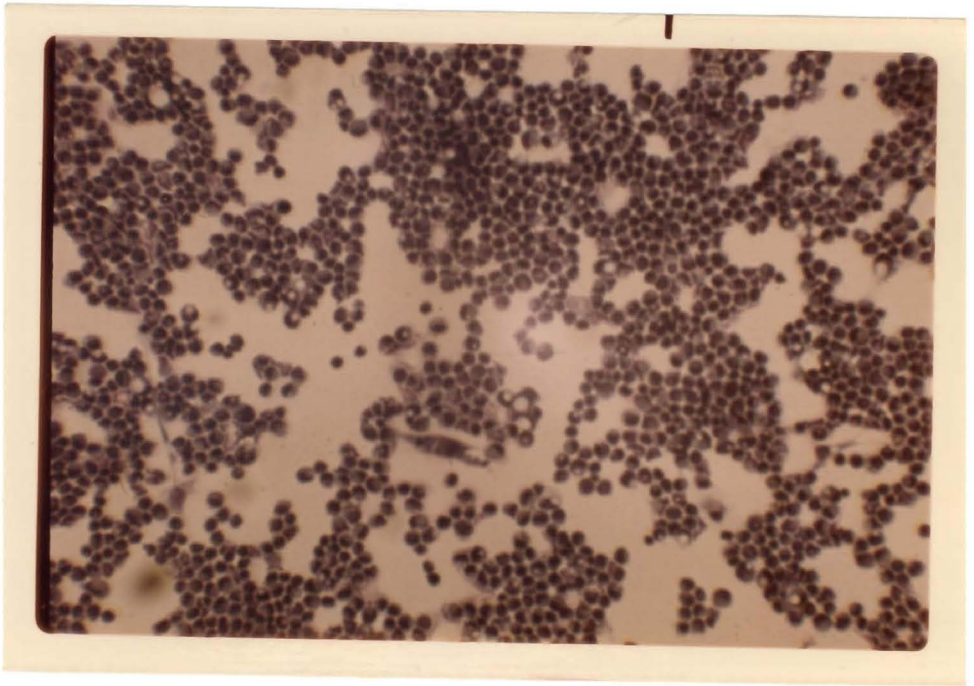
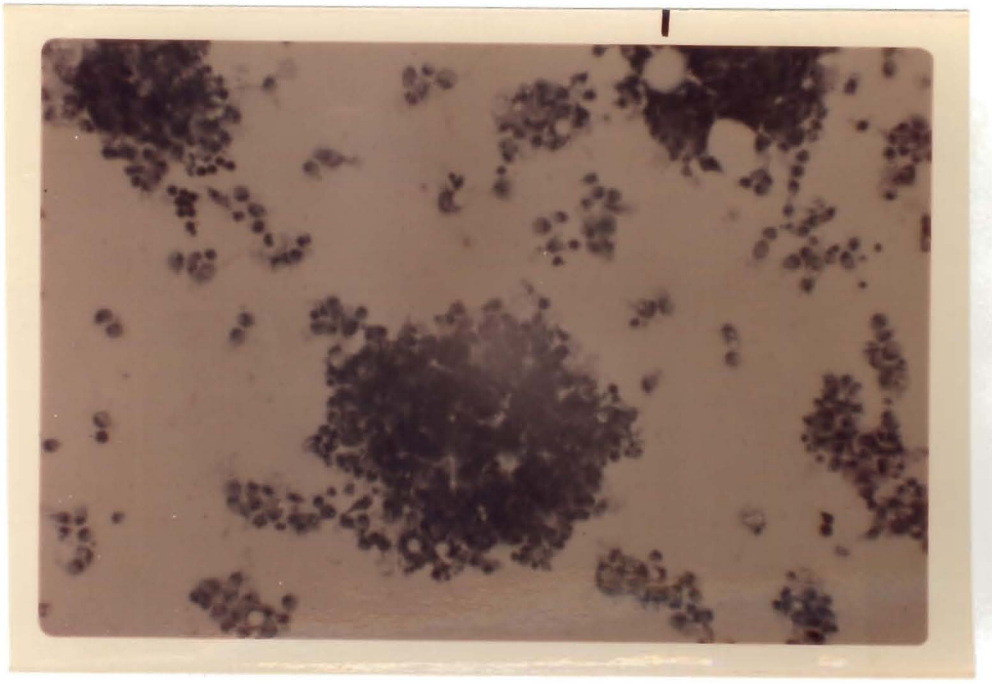


Figure 36: Nonimmune rabbit peritoneal macrophages infected for 72 hours with IHD vaccinia virus. (160X)

Figure 37: Immune rabbit peritoneal macrophages infected for 72 hours with IHD vaccinia virus. (160X)



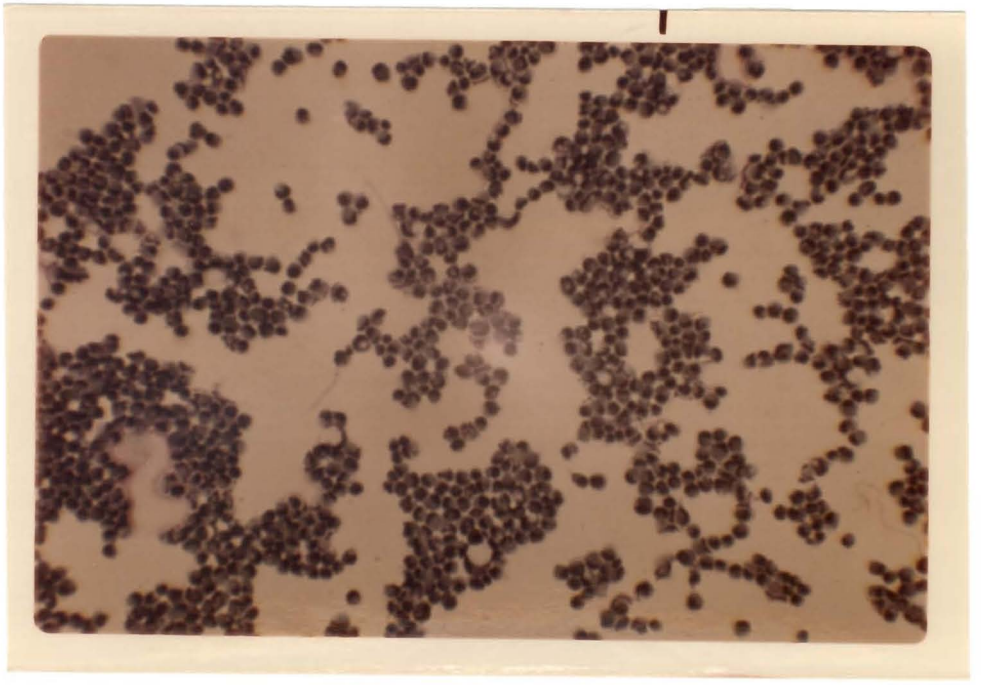
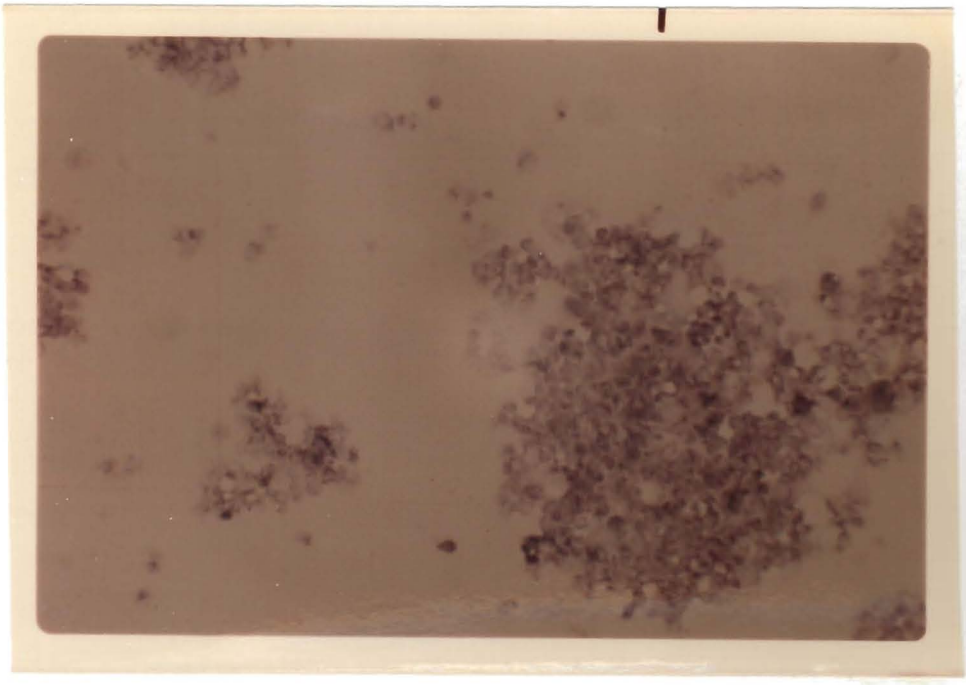


Figure 38: Nonimmune rabbit peritoneal macrophages infected for 120 hours with IHD vaccinia virus. (160X)

Figure 39: Immune rabbit peritoneal macrophages infected for 120 hours with IHD vaccinia virus. (160X)



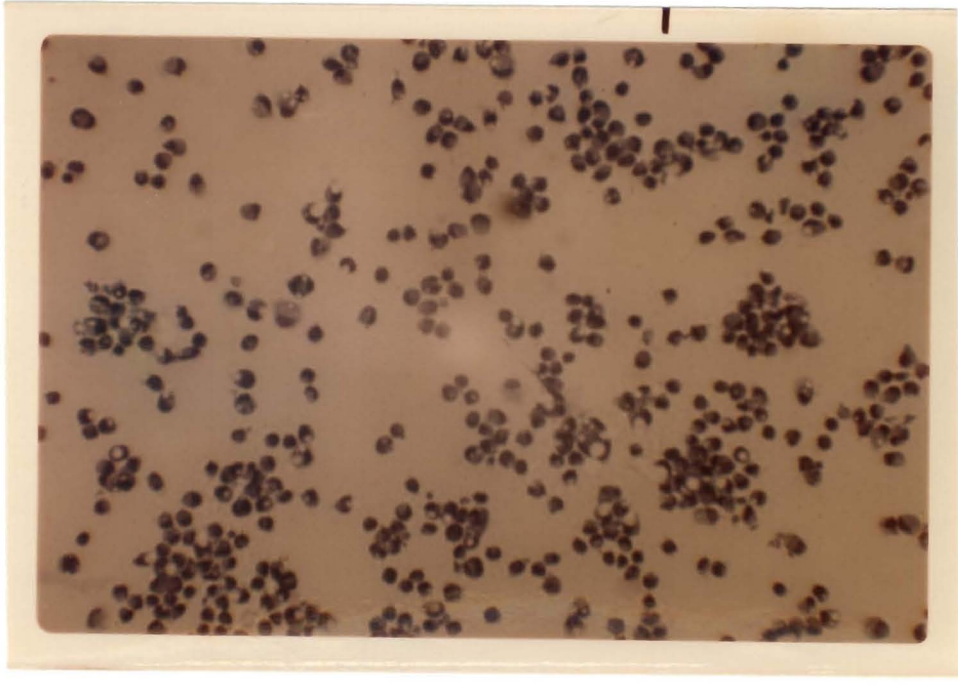


Figure 40: Electron micrograph of nonimmune rabbit peritoneal macrophage two hours post infection. This cell contains virus particles (v) within a phagocytic vacuole. Lead stain; 45,000X.

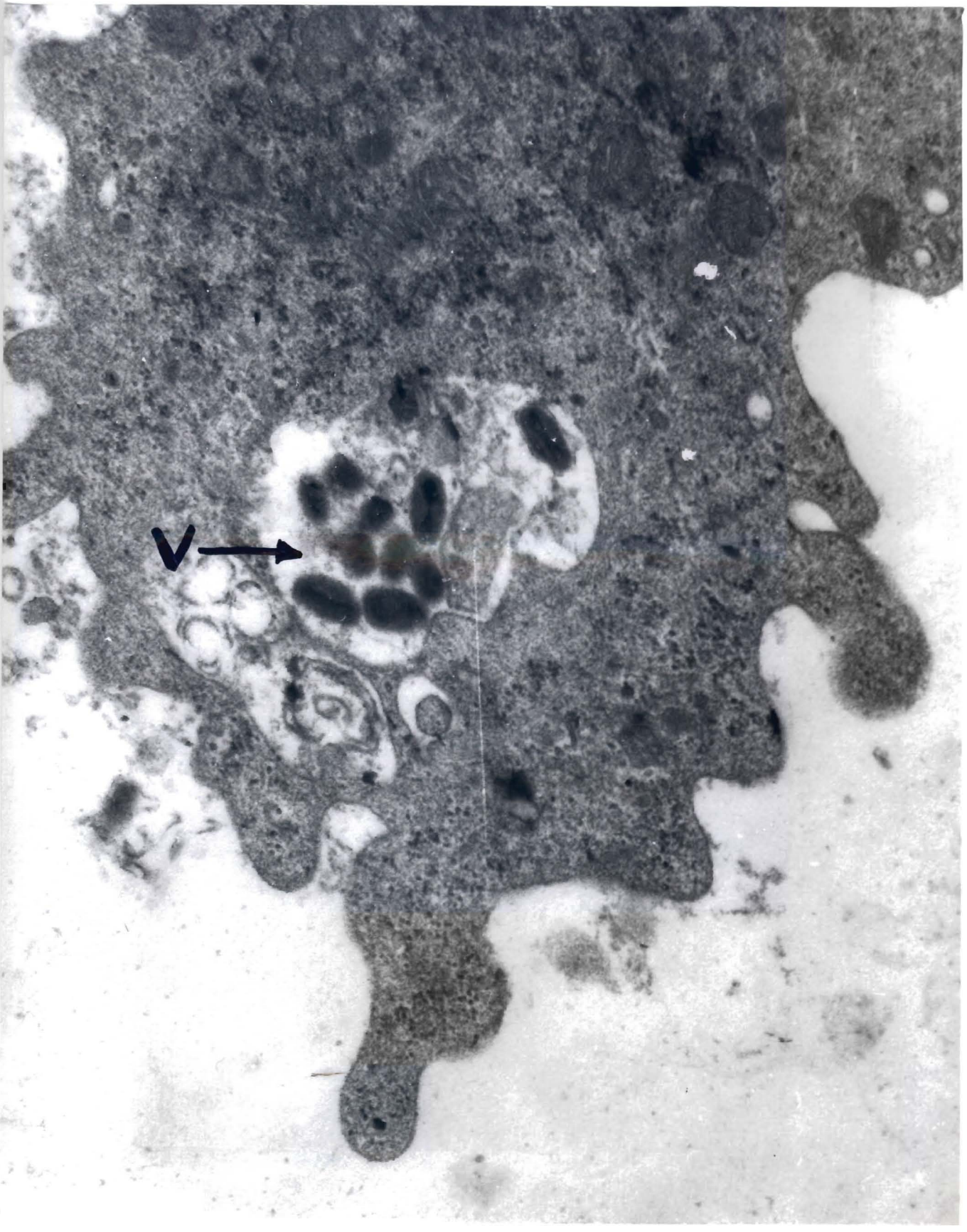
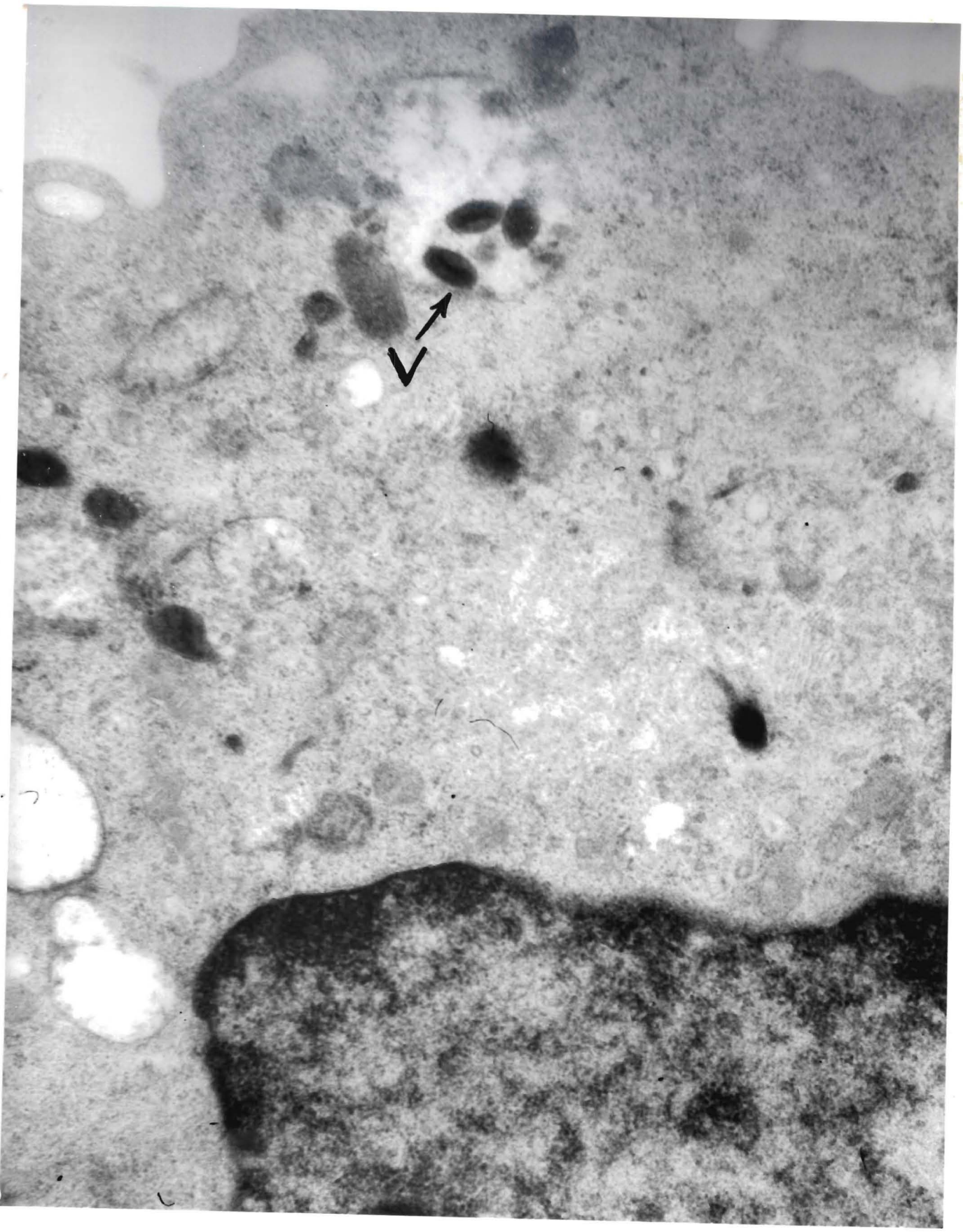

V

Figure 41: Electron micrograph of immune rabbit peritoneal macrophage two hours post infection. This cell contains virus particles (v) within a phagocytic vacuole. Lead stain; 24,000X.



Upon examination of the cellular contents, viral cores were found within the cytoplasm of nonimmune infected macrophages (Fig. 42). This was an indication that first stage uncoating had occurred, removing the outer membrane of the virus, releasing the DNA containing core. Viral cores were also found in the immune cells' cytoplasm (Fig. 43) indicating that first stage uncoating of the virus occurs in immune macrophages.

Since both the nonimmune and immune macrophages could initiate steps toward viral replication, cells infected (0.1 PFU/cell) with virus were examined for evidence of viral replication. Nonimmune infected macrophages examined at 24, 48, and 72 hours contained immature virus particles indicative of viral replication (Fig. 44). A larger percentage of the nonimmune cells contained viral particles with the increase in incubation time.

Immune infected macrophages were examined at 24, 48, and 72 hours. No viral particles were found in the immune cells. There was no evidence of viral replication or cell damage. The appearance of the immune infected cells was that of typical rabbit macrophages (Fig. 45).

Figure 42: Electron micrograph of nonimmune rabbit peritoneal macrophage two hours post infection with viral cores (vc) in the cytoplasm. Lead stain; 42,000X.

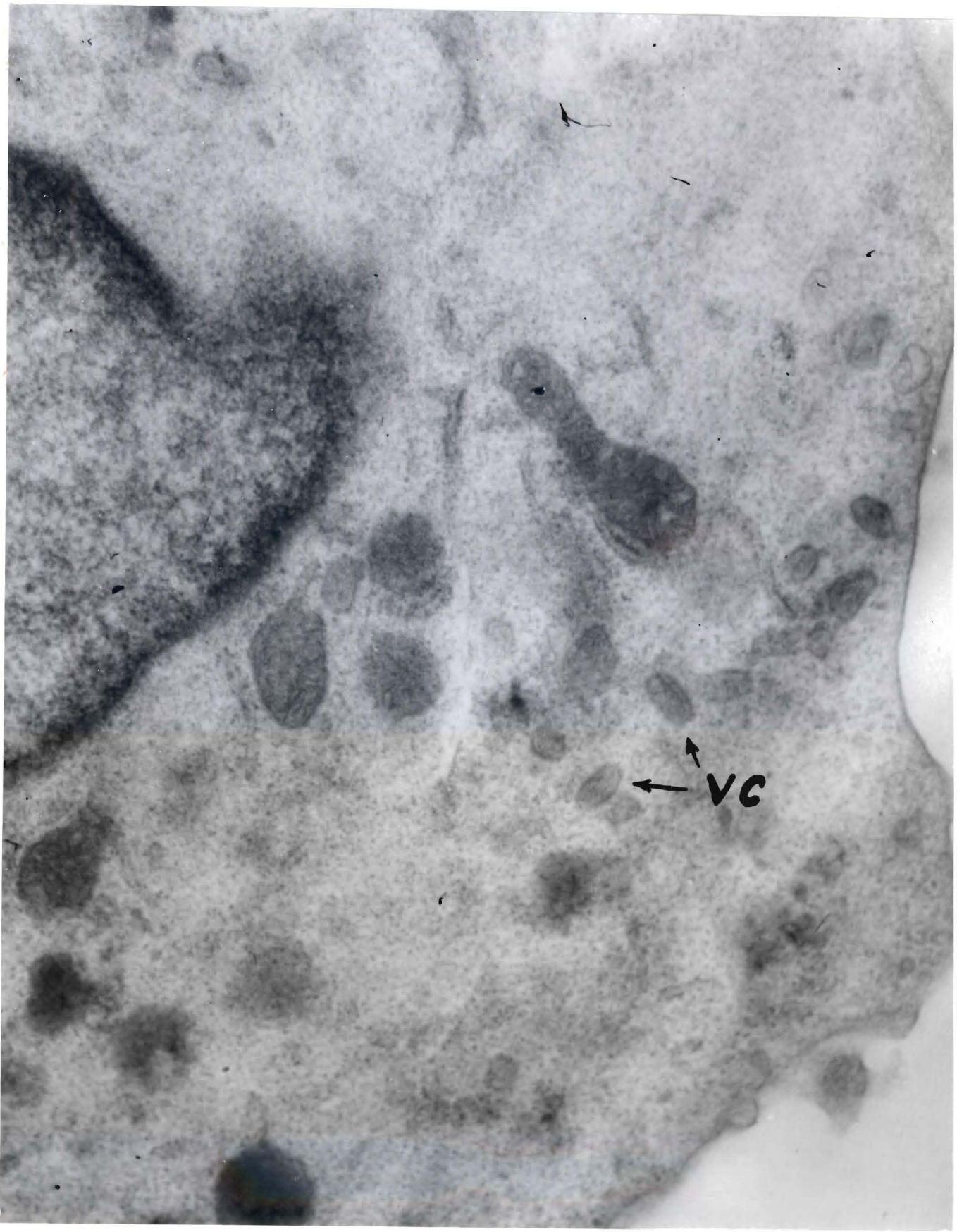

VC

Figure 43: Electron micrograph of immune rabbit peritoneal macrophage two hours post infection with a viral core (vc) within the cytoplasm. Lead stain; 57,000X.

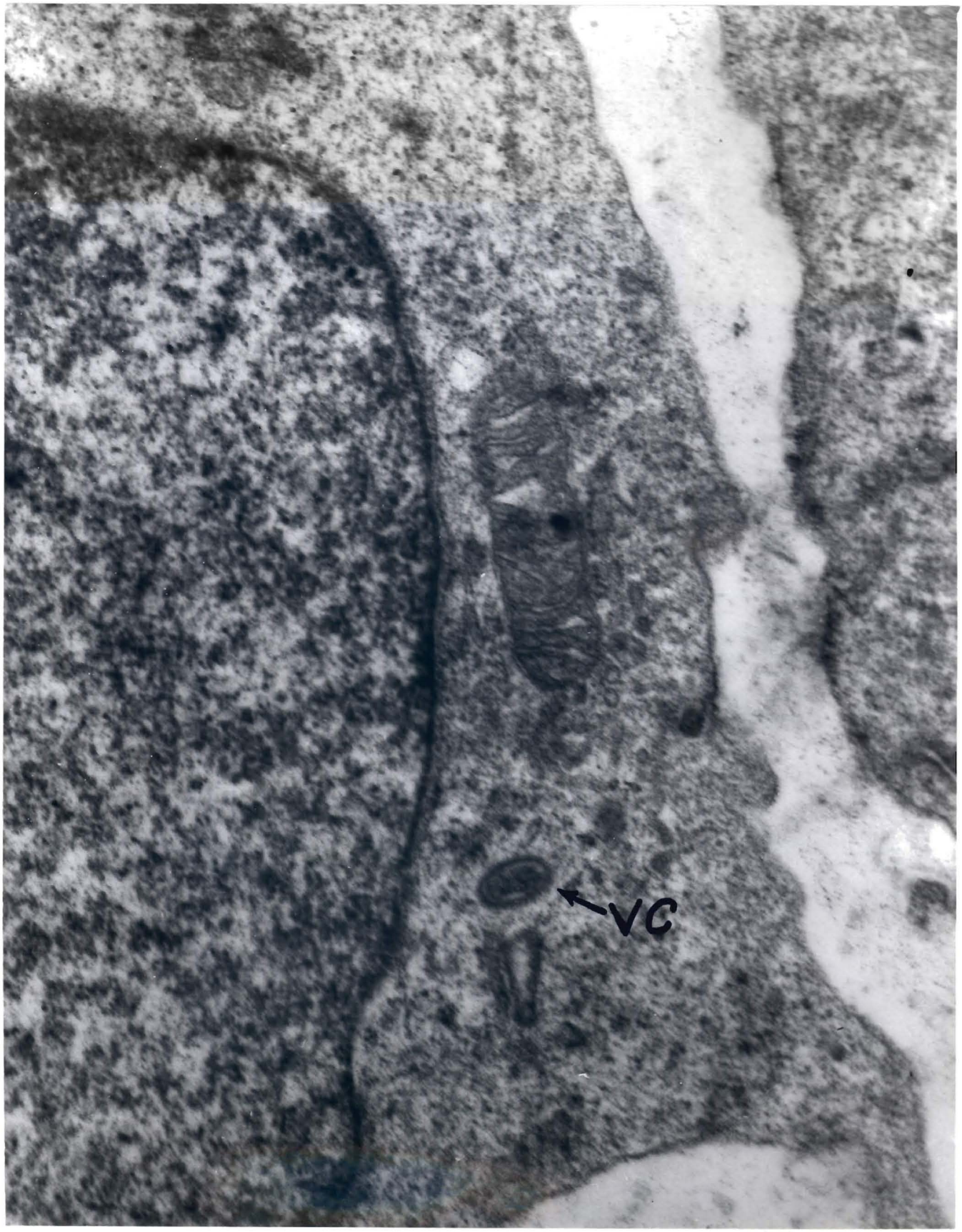

VC

Figure 44: Electron micrograph of nonimmune rabbit peritoneal macrophages infected with IHD vaccinia virus for 72 hours. Developing virus (DV) as well as mature virus (v) are visible. Lead stain; 24,000X.

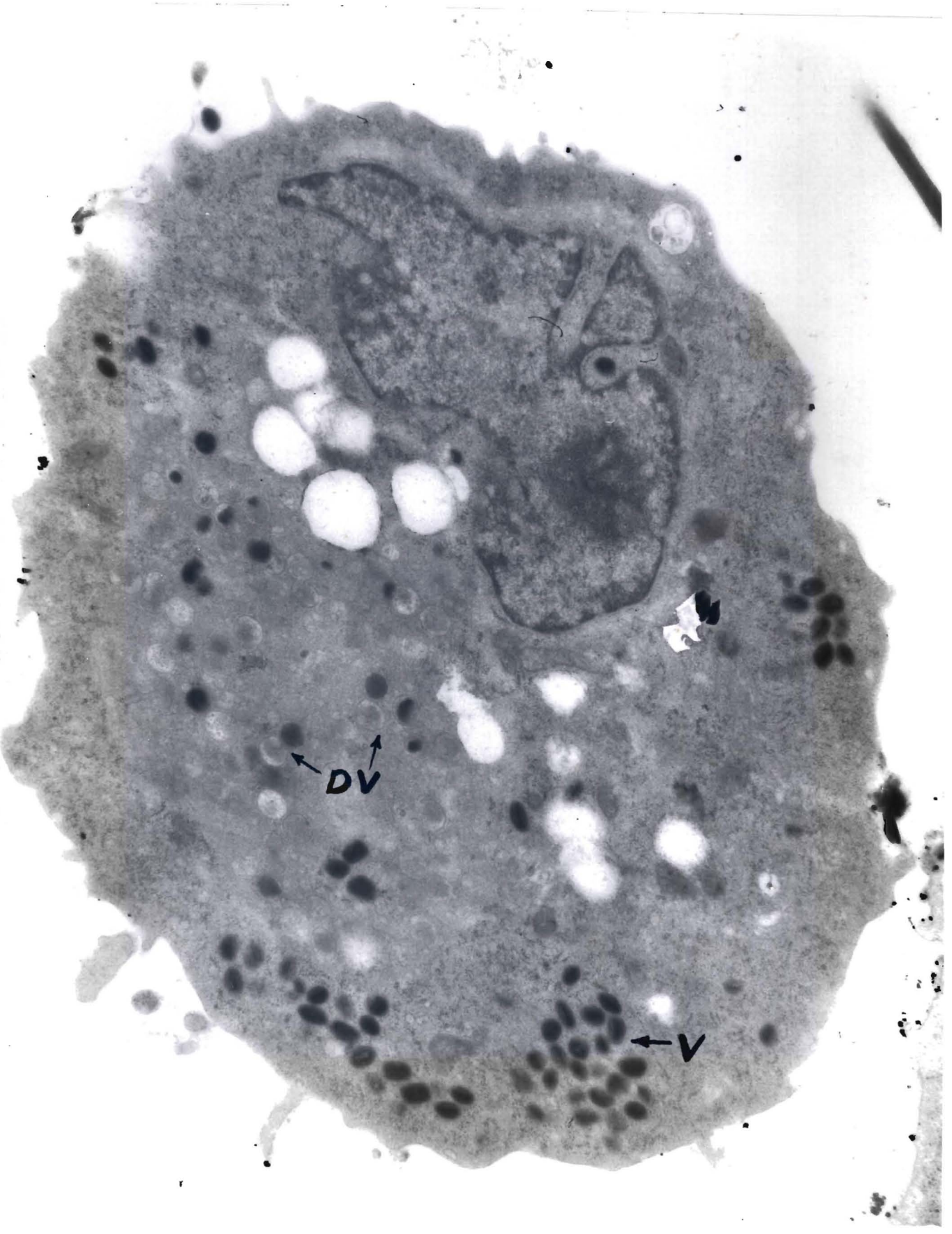
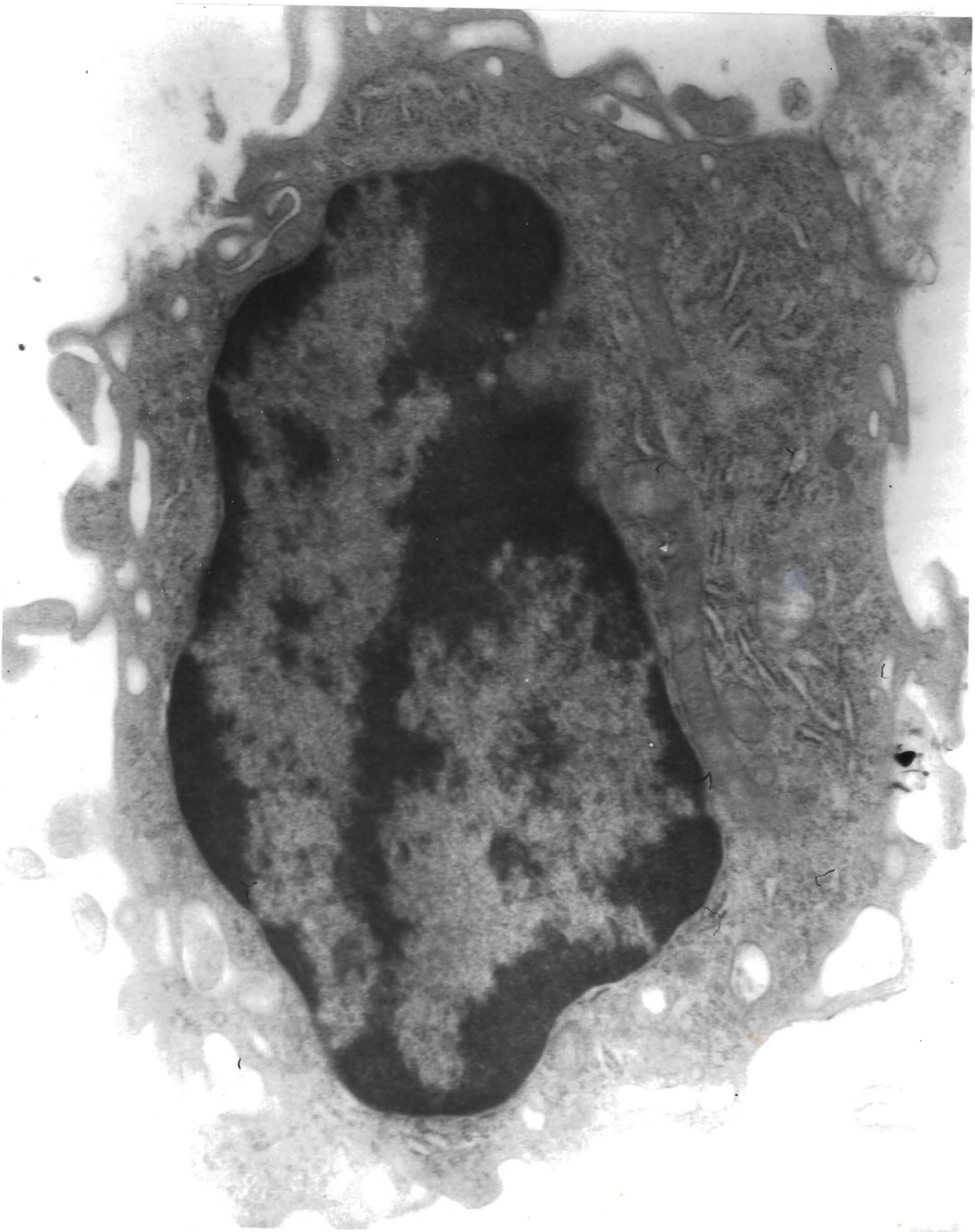

DV
V

Figure 45: Electron micrograph of immune rabbit peritoneal macrophage infected with IHD vaccinia virus for 72 hours. There are no visible signs of viral replication or cell damage. Lead stain; 24,000X.



DISCUSSION

The course of viral infection in an animal may depend on the results of the initial virus-macrophage encounter. Studies concerning abortive and successful viral infections utilizing monocytes and macrophages have indicated that macrophages play an important role in host susceptability and viral pathogenesis. The *in vitro* study of viruses with macrophages has proven to be highly informational as well as less complicated than an *in vivo* study but not without restrictions in regard to implications.

In order to obtain enough peritoneal macrophages for study, stimulation is required of some animals including the rabbit. The injection of mineral oil into the peritoneal cavity has a stimulatory and inflammatory effect facilitating the recovery of large quantities of cells. It has been shown that epithelial and giant cells arise from inflammatory macrophages. During the course of a granulomatous response or under *in vitro* culture conditions (Sutton and Weiss, 1966), the highly phagocytic macrophage undergoes nuclear and cytoplasmic changes (Dumont and Sheldon, 1965). In many cases the nucleus becomes elongated. Slim cytoplasmic extensions are formed and these are intertwined with adjacent epithelioid cells. In addition, there seems to be a change in the relative distribution of cytoplasmic organelles with more mitochondria being apparent and fewer dense bodies. This may be the result of a decrease in endocytic activity and/or the more vigorous formation of mitochondria.

In this study, after prolonged *in vitro* incubation (144 -192 hours) of rabbit macrophage cultures, the predominating cell type was that of epithelial morphology. It was found that the vaccine strain of vaccinia

virus was able to infect and replicate in this cell type much more readily than in rabbit macrophages. In rabbit macrophages there was a delay before replication was evidenced. However, in the "transformed" cultures evidence of replication was seen at 24 hours with accompanying CPE. The CPE became extensive upon further incubation of "transformed" cells. This was in contrast to only slight CPE in vaccine vaccinia infected rabbit macrophages. Therefore, the rabbit macrophages after 144-192 hours of incubation gave rise to an epithelial cell type that was more susceptible to the vaccine strain of vaccinia virus. This effect may be strain specific as the IHD strain replicated readily in rabbit macrophages. The observation was made that the susceptibility of rabbit macrophages to vaccinia virus is strain dependent.

Studies have been conducted altering macrophage function, varying macrophage culture conditions, and determining the effect of various factors on macrophages. Recent work has shown that it is possible to produce selective impairment of macrophage function by silica particles, which are taken up by and damage macrophages (Allison, Harington, and Birbeck, 1966). Macrophage activity has been shown to be altered by antimacrophage serum (AMS). There has been shown to be a marked inhibition of phagocytosis by live macrophages treated with AMS probably as a consequence of the membrane alteration by the antibody. Macrophages treated with AMS, then subjected to complement, resulted in death of the macrophage (Unanue, 1968).

In mice, herpes virus antigen is largely limited to peritoneal macrophages following intraperitoneal inoculation (Zisman, Hirsch, and Allison, 1970). Thus the macrophage is a barrier against the virus.

AMS and silica, which alter macrophage activity *in vitro*, increase mortality from herpes virus infection due to this same alteration of macrophage function *in vivo*.

Methods of impairing macrophage function were investigated in this study. The use of sodium fluoride has been shown to impair phagocytosis. Dales and Kajioka (1964) found that the uptake of vaccinia virus by L cells was prevented upon addition of NaF to the cells. Therefore, inhibition of phagocytosis in macrophages by the addition of NaF was investigated. Different concentrations, which have been reported to stop phagocytosis in other cell types, were tried. However, phagocytosis of bacteria by macrophages was observed at all concentrations used. The NaF proved to have a highly toxic effect on macrophages and therefore could not be used to prevent phagocytosis by macrophages.

Low temperatures of incubation have been an approach used to shed light on the discriminative powers of macrophages by differentiating those factors required for specific attachment from those required for the cell to engulf the particle (Brumfitt *et al.*, 1965). In this study, it was found that cold (4° C) temperature stopped phagocytosis of bacteria by rabbit macrophages. Vaccinia virus added to macrophages at 4° C attached firmly enough to resist washing. After removal of residual virus, washing, replacing with fresh medium, and incubating at 37° C, cells infected in this manner supported replication of the virus. These findings were in line with the suggestion of Silverstein (1970) that 4° C temperature allows attachment of virus to cells but slows down or almost stops phagocytosis by macrophages.

Certain substances are capable of interacting with and altering the metabolism of cells. It has been shown that Con A, a lectin, will react with surface receptors of macrophages, altering the metabolism of these phagocytes (Romeo, Zabucchi, and Rossi, 1973). The possibility that the alteration of metabolism might affect viral interaction with macrophages was investigated in this laboratory. Macrophages obtained from mice stimulated with Con A four days prior to harvest handled IHD vaccinia virus in a manner almost identical to Eagle's MEM stimulated or unstimulated mouse macrophages. Con A stimulated macrophages were capable of effecting a decline in infectious viral titer with increased incubation as were normal mouse macrophages. Therefore, the metabolism of the macrophages was not detectably altered in regard to viral handling.

The results of various lines of experimentation have implicated receptor sites, interferon, cytophilic antibody, and unbound antibody as being responsible for the interaction of viruses with macrophages. Information is not complete on the cell surface receptors with which particles interact during the initial stages of phagocytosis. Whether specific macrophage cell receptors exist for viruses is not known. Some particles require no serum factors for uptake by phagocytes, whereas others must be coated with heat labile complement-like materials or with specific antibody (Hirsch and Strauss, 1964).

Wright and Douglas (1904) showed that sera from nonimmunized animals contained molecules which react with bacteria to make them more susceptible to phagocytosis by phagocytic cells. Serum influenced phagocytosis by altering the particle to be phagocytized, and not by acting on the cell.

Wright and Douglas (1904) called the responsible serum components opsonins and envisaged that they combine with and "coat" bacteria.

In this study, the effect of the presence of serum on uptake of vaccinia virus by rabbit macrophages was investigated. The presence of serum allowed for the uptake of approximately five times more virus than without the presence of serum. However, virus was able to attach to macrophages in a significant amount in the absence of serum. It is not known if the presence of serum had an effect on phagocytosis. Perhaps the virus attached without the presence of serum but was not phagocytized. After adsorption without serum, medium containing serum was added; therefore virus attached to the macrophages could then enter the cell if serum were necessary for phagocytosis. Electron microscopy could be used to determine the effect of serum on phagocytosis of vaccinia virus.

Two types of serum were investigated in regard to infection of macrophages with vaccinia. Vaccinia virus replicated in rabbit macrophages equally well when medium was supplemented with bovine or fetal bovine serum. There did not appear to be substances in either sera that were inhibitory or promotional for vaccinia replication.

The amount of serum present was not found to be a factor in viral replication. A concentration of 10% serum was necessary to maintain macrophages in good condition. This amount of serum was adequate for the purpose of viral replication as an increase in serum content to 30% did not significantly alter the pattern or amount of replication.

Rabinovitch (1967) has suggested that specific receptors exist at the cell surface for various particles and has shown that treatment of

phagocytes with trypsin can reduce the interaction of gluteraldehyde treated erythrocytes (GRC) with phagocytes. Therefore, in this study trypsin treatment was used on rabbit peritoneal macrophages prior to addition of infecting vaccinia virus to determine if this treatment altered the attachment and replication of virus in macrophages. It was found that trypsin treatment did not drastically alter the amount of virus adhering to the macrophages. Following attachment the virus replicated in trypsin-treated macrophages in a manner only slightly different than in untreated macrophages. The amount of virus produced in the trypsin treated macrophages was comparable to the amount of virus produced in untreated rabbit macrophages. These results lead to one of two possibilities; vaccinia virus receptors are not affected by trypsin, or receptors are not necessary for the uptake of vaccinia virus.

The importance of opsonins in acquired immunity is evidenced by the fact that specific antisera are able to promote phagocytosis at a much greater dilution than normal serum (Mudd et al., 1930). The way in which serum opsonins stimulate phagocytic mechanisms is unknown. Presumably the opsonins serve as recognition factors for phagocytic cells. Studies with ^{3}H thymidine labeled vaccinia virus indicate that particles neutralized with hyperimmune rabbit serum are ingested more avidly than untreated control virus (Silverstein, 1970). In this laboratory virus preincubated with immune rabbit serum was added to rabbit macrophages. The amount of infectious virus was reduced by this incubation; also the amount of infectious virus taken up by the macrophages was reduced. This result was expected, as antibody neutralized virus is no longer infective. Therefore, using an assay of infectious virus, it was not possible

to determine if antibody neutralized vaccinia was more avidly phagocytized than untreated vaccinia. After infection of macrophages with antibody neutralized virus, no CPE was observed and infectious virus was not recovered.

One explanation for the mechanism of macrophage activation postulates a role for cytophilic antibody. Cytophilic antibodies have been defined as globulin components of antiserum which become attached to certain cells in such a way that these cells are subsequently capable of adsorbing antigen (Boyden, 1964). This antibody is said to constitute no more than one per cent of the free antibody of serum. The production of cytophilic antibodies to antigens other than sheep erythrocytes has not been investigated in detail.

Thus, the possibility of cytophilic antibody production in the immunization of rabbits with vaccinia virus was investigated. The treatment of rabbit macrophages with immune serum before the addition of virus produced no observable difference in the amount of virus adsorbed by these cells and the subsequent replication of virus. However, there are various factors that govern the production of cytophilic antibodies. It has been found that production of cytophilic antibodies in the guinea pig is dependent on the route of injection and the amount of time after immunization (Gowland, 1966). Therefore, the optimal conditions for obtaining cytophilic antibodies in rabbit serum would have to be determined.

The discovery in 1957 by Isaacs and Lendenman of interferon revealed for the first time that animals had, in addition to antibody, a second form of acquired immunity. The first interferon described was secreted

by cells that had been exposed to inactivated virus. Absorption of the interferon protected the adjacent cells against a subsequent exposure to active virus. Glasgow (1966) found that mouse peritoneal cells treated with Chikungunya virus produced more interferon than those treated with vaccinia virus. Macrophages from mice immunized with Chikungunya virus produced more interferon upon exposure to this virus than did macrophages from nonimmune mice. Furthermore the enhanced production of interferon resulted only when cells were challenged with Chikungunya virus, suggesting that macrophages may possess a specific "immunological memory".

Enhanced production of an interferon-like substance by immune macrophages does not appear to be the cause of suppression of vaccinia virus replication as only low levels of interferon-like substances were found (Tompkins, Zarling, and Rawls, 1970). There appeared to be no difference between titers of interferon-like activity from nonimmune and immune cells infected with vaccinia. These results are consistent with those of Subrahmanyan and Mims (1970) who were unable to induce interferon with vaccinia virus in macrophages from either immune or nonimmune mice.

The possibility of cell-bound antibody to vaccinia virus also appears to be an unlikely explanation for the inhibition of vaccinia replication in immune macrophages since no vaccinia neutralizing activity was found associated with heat eluates or whole-cell extracts of sensitized macrophages (Tompkins, Zarling, and Rawls, 1970).

Vaccinia immune and nonimmune rabbit macrophages adsorb equal amounts of virus. It was found in this study that immune macrophages could phagocytize vaccinia virus, and that the virus did not remain at the cell surface as proposed by Avila, Schultz, and Tompkins (1972).

Upon examination of vaccinia infected immune macrophages with the electron microscope, viral particles were seen within vacuoles, establishing phagocytosis. Viral cores were found within the cytoplasm of infected immune cells. Therefore, the immune cells were capable of processing vaccinia through the first stage of uncoating.

Immune and nonimmune cells handle the virus initially in like manner; i.e., attachment, phagocytosis, and first stage uncoating of the virus. Vaccinia undergoes a two stage uncoating process in order to initiate viral replication (Dales, 1965). It is not known if second stage uncoating occurs in the immune cells. However, there was no evidence of any viral replication in immune infected cells examined by electron microscopy. It seems feasible that the lack of replication in immune cells is due to some occurrence following first stage uncoating.

SUMMARY

Infection of normal rabbit macrophages with vaccinia virus leads to viral replication. It was shown that there are differences in strains of vaccinia virus evidenced by the variance in amount and manner of replication and observable CPE. The IHD strain was shown to replicate more readily in and destroy rabbit macrophages than the vaccine strain. The vaccine strain, however, replicated more fully in "transformed" rabbit macrophages than in rabbit macrophages.

The IHD strain of vaccinia does not replicate in mouse macrophages. The fate of the virus was unchanged in Con A stimulated mouse macrophages. Trypsin treatment of rabbit macrophages did not significantly alter the uptake and replication of IHD vaccinia virus within these cells.

Serum was shown not to be essential for the attachment of virus to macrophages. However, the presence of serum slightly increased viral adsorption. The concentration of serum was found not to be a factor in replication. Bovine and fetal bovine sera equally supported vaccinia virus replication in macrophages.

Immune serum applied to the virus neutralized vaccinia and prevented viral replication in rabbit macrophages, whereas immune serum applied to rabbit macrophages did not contain cytophilic antibody in a significant amount to alter the uptake or replication of virus.

Macrophages obtained from rabbits immunized with vaccinia virus failed to support replication of the virus. Immune and nonimmune macrophages took up equal amounts of virus. Both immune and nonimmune cells were observed, using the electron microscope, to phagocytize the

virus and to be capable of first stage uncoating of the virus.

The basis for the lack of viral replication in immune rabbit macrophages is unknown. It seems plausible that some malfunction in the replication sequence occurring after first stage uncoating is responsible for the lack of replication.

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