INTRACELLULAR MECHANISMS OF INNATE RESISTANCE TO VACCINIA VIRUS

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Previous studies have demonstrated that mouse peritoneal macrophages restrict vaccinia virus replication in vitro, whereas rabbit peritoneal macrophages are susceptible. Previous autoradiography experiments revealed that no significant viral DNA synthesis appeared in mouse macrophages, indicating that an abortive infection had occurred which blocked viral replication prior to DNA synthesis. In an attempt to define the nature of this restriction, an analysis of the virus infectious cycle was undertaken.

Electron micrographs revealed that adsorption, first stage of uncoating, and second stage of uncoating were identical in both mouse and rabbit macrophages. Assembly of viral components was detected in rabbit macrophages but not in mouse macrophages. Biochemical studies revealed no thymidine phosphorylating activity in mouse macrophages indicating that the block in the virus replicative cycle occurred at the narrow margin between the second stage of uncoating and viral DNA synthesis. These data support our hypothesis that normal mouse peritoneal macrophages contribute to specific, non-interferon mediated resistance to vaccinia virus by aborting the infection at some early stage of the replicative cycle.

TABLE OF CONTENTS

PAGE

LIST OF TABLES vii

LIST OF FIGURES. viii

INTRODUCTION 1

MATERIALS AND METHODS. 10

Experimental Animals. 10

Cell Culture Media. 10

Cell Culture Procedure. 10

Mouse Fibroblast Cultures. 11

Mouse Peritoneal Macrophages 11

Rabbit Peritoneal Macrophages. 12

Staining of Cells on Coverslips 13

Vaccinia Virus. 13

Virus Propagation. 13

Harvesting of Virus. 14

Virus assay. 14

Purification of labeled virus. 15

Electron Microscopy 15

Preparation of Peritoneal Macrophages. 15

Cell Culture Infection 16

Cell Processing. 16

Film Processing. 17

Biochemical Studies 18

Assay of Second Stage Uncoating. 18

Thymidine Kinase Activity. 18

RESULTS 20

Electron Microscopy. 33

Attachment. 33

Viral phagocytosis. 33

First stage of uncoating. 33

Viral assembly. 48

Biochemical Tests. 67

Second stage of uncoating 67

Thymidine kinase. 67

DISCUSSION. 72

SUMMARY 77

LITERATURE CITED. 79

LIST OF TABLES

TABLE		PAGE
1	Number of viral cores per 150 sections of mouse and rabbit macrophages with respect to time	50
2	Number of areas of viral reproduction per 100 sections of mouse and rabbit macrophages.	66
3	Assay of the second stage of uncoating in rabbit macrophages at 30 min, 4 h, and 8 h postinfection	69
4	Assay of the second stage of uncoating in mouse macrophages at 30 min, 4 h, and 8 h postinfection	69

LIST OF FIGURES

FIGURES		PAGE
1	Mouse peritoneal macrophage (X 100) May Grunwald-Giemsa stain.	22
2	Rabbit peritoneal macrophage (X 100) May Grunwald-Giemsa stain.	24
3	Infected rabbit peritoneal macrophages at MOI of 1.0 after 72 h incubation. (X 400) May Grunwald-Giemsa stain.	26
4	Infected mouse peritoneal macrophages at MOI of 1.0 after 72 h incubation. (X 400) May Grunwald-Giemsa stain.	28
5	Electron micrograph of a rabbit peritoneal macrophage after 8 h incubation. Several mitochondria and cytoplasmic vescicles of varying sizes are seen. (X 38,000) Uranyl acetate/lead citrate stain.	30
6	Electron micrograph of a mouse macrophage after 10 h incubation. Cytoplasmic vescicles and phagocytic vacuoles are seen. (X 38,00) Uranyl acetate/lead citrate stain.	32
7	Electron micrograph of infected mouse peritoneal macrophage at zero h showing vaccinia virus in close proximity. (X 48,000) Uranyl acetate/lead citrate stain.	35
8	Electron micrograph of infected mouse macrophages at zero h showing attachment and phagocytosis of vaccinia virus. (X 36,000) Uranyl acetate/lead citrate stain.	37
9	Electron micrograph of infected rabbit peritoneal macrophage at 0 h showing virus in close proximity. (X 54,000) Uranyl acetate/ lead citrate stain	39
10	Electron micrograph of infected rabbit macrophage after 3 h showing phagocytosis and 3 viral cores in the cytoplasm. (X 54,000) Uranyl acetate/lead citrate stain.	41
11	Electron micrograph of infected rabbit macrophage after 3 h showing a viral core in the cytoplasm. (X 54,000) Uranyl acetate/lead citrate stain.	43

12 Electron micrograph of infected mouse peritoneal macrophage showing a viral core and its spike in the cytoplasm. (X 54,000) Uranyl acetate/lead citrate stain. 45

13 Electron micrograph of infected rabbit peritoneal macrophage after 6 h. The beginning of viral synthesis is seen. (X 48,000) Uranyl acetate/lead citrate stain. 47

14 Electron micrograph of infected mouse peritoneal macrophage after 6 h. (X 46,000) Uranyl acetate/lead citrate stain. 52

15 Electron micrograph of rabbit peritoneal macrophage at 12 h after infection. Immature virus can be seen. (X 54,000) Uranyl acetate/lead citrate stain. 54

16 Infected mouse peritoneal macrophage after 12 h incubation. (X 54,000) Uranyl acetate/lead citrate stain. 56

17 Infected rabbit peritoneal macrophage after 12 h incubation. Immature forms of vaccinia virus are evident in viroplasm. (X 56,000) Uranyl acetate/lead citrate stain. 58

18 Electron micrograph of infected mouse macrophage after 24 h incubation. (X 54,000) Uranyl acetate/lead citrate stain. 60

19 Electron micrograph of infected rabbit macrophage after 24 h incubation. Maturing particles of vaccinia virus, some showing more condenced DNA (v), can be seen in the cytoplasm. (X 54,000) Uranyl acetate/lead citrate stain. 62

20 Infected mouse peritoneal macrophage after 24 h incubation. (X 54,000) Uranyl acetate/lead citrate stain. 64

21 Thymidine kinase activity in mouse and rabbit macrophages infected up to 24 h with IHD vaccinia virus. 71

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INTRODUCTION

In analyzing host defense mechanisms against virus infections, two aspects have to be considered: acquired immunity and innate resistance. The first aspect concerns defense mechanisms that the host displays specifically against the inducing virus. Specific host responses allow recovery from a fully established infection and provide long term protection against infection with the same or an antigenically closely related virus. The second aspect, which has been variously called natural or non-specific resistance, concerns defense developed through inheritance and normally passed on to new generations. Natural immunity is not conferred by immunological mechanisms, rather it represents a species-specific characteristic.

Compared with acquired immunity, very little is known about resistance in natural genetically determined host defense against viruses. Inborn resistance can be a function of physical and anatomical barriers at any point(s) in the virus-host interaction. Depending on the system studied, the following possibilities have been considered: lack of cell surface receptors (Merigan 1974), release of non-infectious virus, presence of humoral inhibitors in serum or secretions (Smith 1972), induction of interferon (Isaacs and Lindemann 1957, Baron 1973), triggering of cellular, humoral and secretory immune response (Mogensen 1979), peculiarity of macrophages (Mims 1964), fever, pH (Baron 1963), and activation of natural killer cells. This research thesis focuses on phagocytosis by mouse macrophages and the role of this specialized cell function in host protection against vaccinia virus.

Eli Metchnikoff (1892) was the first to introduce the term macrophage and to recognize the capacity of this cell to ingest foreign material. In addition to the proficiency at phagocytosis, macrophages possess the ability to process antigens for the immune response (Roitt 1985) and to secrete a variety of biologically active molecules (Hood 1978).

Macrophages are derived from bone marrow stem cells. Immature macrophages are released into the blood stream where, as monocytes, they account for 5 % of the total blood leukocyte population (Tigard 1986). After circulating for several days, monocytes migrate into the various organs and tissue systems and mature into macrophages. These become the Kupffer cells of the liver; histiocytes of connective tissue, free and fixed macrophages of the spleen, lymph nodes, and bone marrow; alveolar macrophages of the lungs, and macrophages monitoring serous spaces, such as the pleural and peritoneal cavities.

The cytology and physiology of macrophages vary depending on their habitat. In suspension they are round cells, about 15 to 20 micrometers in diameter. They have a bean shaped to round nucleus and a cytoplasm rich in mitochondria, lysosomes, and rough endoplasmic reticula. They also possess a Golgi apparatus. Ruffled cytoplasm, active phagocytosis and pinocytosis and rapid attachment to glass surfaces are common characteristics of these mononuclear phagocytic cells (Van Furth 1975).

Macrophages monitor the main body compartments; consequently, they often encounter infecting virus particles, usually at the initial stage of the viral infection (Mims 1964). They are in a position to control the susceptibility of animals to virus infections as they can

interfere with the early stages of virus invasion, multiplication, and spread to target organs. This is why they are thought to represent a first line of antiviral defense. In the liver, for instance, Kupffer cells lining the sinusoids constitute an intact barrier protecting the adjacent parenchymal cells (Haller 1980). Infection of Kupffer cells often represents the key event of the pathogenesis of an infectious disease (Kirn and Keller 1983).

Heredity plays an important role in the resistance to mammalian viral infections (Gowens 1948). Evidence for macrophage involvement in this respect, however, was slow to develop. In fact, some early studies tended to support the view that antibody is necessary for recovery. Overman and Kilham (1953) found that young hamsters developed lethal infections from mumps encephalitis only through the age of six days. They correlated lethal infections with a prolonged time for antibody formation in the young hamsters in comparison to the resistant older hamsters. Using ectromelia-susceptible and resistant mice strains, Schell (1960), found that antibody response of resistant mice occurred before that of the susceptible mice. These studies correlated the time of antibody response with the ability to recover from infection.

Recent evidence, however, supports the concept that immune mechanisms may not be essential for the elimination of viruses from the infected animal. Particularly important are the findings that production of antibodies <u>in vivo</u>, even at high titres, does not result in the elimination of simian viruses (Meyer et al. 1962), polyoma virus (Habel and Silverberg 1960), adenoviruses (Rowe et al. 1955) and human immunodeficiency virus (Langone 1986). If the virus of choriomeningitis is injected into suckling mice, the virus develops and

no antibodies are formed. Nevertheless, a state of immunity develops (Lwoff 1959). Furthermore, agammaglobulinemic patients synthesize very small amounts of globulins, and yet they can be immunized against small pox (Gitlin et al. 1959).

Explanation for the inefficiency of antibody in enhancing recovery is obvious when one considers that all viruses multiply intracellularly and are therefore protected from antibody within the host cell. In the case of viruses such as herpes viruses and poxviruses, which can spread directly between cells, infection could not be fully stopped by antibody. In addition, the course of viral infections is not altered in immunologically inhibited animals and passively transferred antibody does not enhance recovery (Baron 1963).

There is good evidence for a protective role of macrophages in viral infections. In his study of age dependent susceptibility of mice to herpes simplex virus (HSV), Johnson (1964) showed that while uptake of virus by adult and suckling mouse macrophages was similar, suckling mouse macrophages tended to allow infection of surrounding cells whereas adult macrophages did not.

Hirsch et al. (1970) showed that transplanting stimulated adult macrophages into syngenic suckling mice protected the latter from infection with HSV. They proposed that the adult macrophage was able to phagocytize and destroy the virus more efficiently than the immature macrophage.

Studies comparing the pathogenic event <u>in vivo</u> and virus-cell interaction <u>in vitro</u> have yielded valuable information on macrophage-virus interaction. In some cases, failure of the macrophage to replicate a virus was correlated with resistance of the animal to that

particular infection.

Bang and Warwick (1957) reported that virulent strains of New castle disease virus destroyed chicken macrophages in vitro, whereas the avirulent strains grew poorly. Likewise, Roberts (1963) showed that avirulent ectromelia virus strains did not infect or replicate in Kupffer cells to the same extent as the virulent stains.

Theis and Koprowski (1961) compared virus yield from West Nile-infected tissue cultures of spleen and peritoneal exudate cells. They found that macrophages from spleens of virus-resistant mice produced little or no virus, but splenic macrophages of virus-susceptible mice released substantial amounts of infectious virus. Similar observations were also made when virus yield from peritoneal macrophages of resistant and susceptible mice were compared. They found that macrophages from susceptible mice immunized against West Nile virus were infected with the virus but, like resistant mouse cultures, they produced little infectious virus. Using fluorescent West Nile antibody, they showed that macrophages from susceptible mice and immunized mice contained the same percentage of cells exhibiting fluorescence, indicating synthesis of viral proteins. In contrast, infected macrophage cultures from resistant mice showed few fluorescing cells.

Perhaps even more interesting were the observations of Sabin (1954) that susceptibility of Webster and Princeton (PRI) strains of mice is determined by a single pair of genes, with resistance being dominant. Bang and Warwick (1960) went on to show, by making the appropriate crosses and back crosses of the two stains, that susceptibility of PRI mice to infection segregated as a single

Mendelian dominant genetic factor. It was suggested that this genetic difference operates at the macrophage level, thus substantiating the role of macrophages in manifesting inherited resistance in vitro, in the absence of acquired immune response.

Replication of poxviruses in macrophages is related to both virus and cell strain. Vaccinia virus, for instance, replicates in non-immune rabbit peritoneal macrophages (Beard et al. 1938; Florman et al. 1942; Greer et al. 1975), but not in normal mouse peritoneal macrophages (Nishmi and Bernkopf 1958; Nishmi and Niecikowski 1963; Glasglow 1965; Schultz 1966; Ward 1980; Kashanchi 1986). Although several in vitro methods have been used in the study of virus uptake and replication in macrophages (Mogensen 1979), little is known about the intracellular events which allow a virus to replicate in the macrophage or the factors determining the macrophages' failure to support replication.

A brief description of vaccinia virus and the early events in its infection of a permissive host cell are outlined below as a reference for subsequent discussion. Vaccinia virus, a member of the poxvirus group (Joklik 1966), is a brick-shaped particle approximately 3000 A X 2000 A x 2000 A (Dales 1965; Joklik 1968). It has a lipoprotein envelope (Dales 1968) which encases a biconcave protein core containing the viral genome, five to ten percent of which is made up of single-stranded DNA. The rest is double stranded. Two proteinaceous structures called lateral bodies lie next to the concave surfaces of the core. The function of these lateral bodies is unknown.

The genome of vaccinia virus encodes information for some 230 different polypeptides of which approximately 100 are incorporated into

the mature virion (Dales and Pogo 1981; Moss 1985). Acrylamide gel analysis indicates that there are at least 17 protein species (Holowezak and Joklik 1967). Except for four enzymatic activities, a DNA dependent RNA polymerase (Isaacs and Lindenmann 1957) a nucleotide phosphohydrolase (Gold and Dales 1968; Munyon et al. 1968), and acid and neutral DNase (Pogo and Dales 1969), the biological activities and functions of most of these proteins are presently unknown.

Vaccinia virus obtains entry into macrophages by endocytic mechanisms (Silverstein 1970). Within the phagosome, the viral envelope interacts with the membrane of the phagosome resulting in the fusion of the two membranes and release of viral cores into the cytoplasmic matrix (Dales 1965; Joklik 1968). This is known as the first stage of uncoating. Immediately following the first stage of uncoating, the so-called early proteins are synthesized. Some of these are thymidine kinase, viral DNA polymerase and alkaline DNase.

After a lag period the disruption of viral cores begins and results in the release of naked viral DNA. This second stage of uncoating is characterized by its dependence on protein synthesis and the sensitization of vaccinia virus DNA to DNase (Joklik 1963). The production of a virus-coded uncoating protein occurs during this lag period.

Studies in this laboratory have concentrated on the vaccinia virus-macrophage model in an attempt to elucidate the mechanism that allows mouse macrophages to abort vaccinia infection. Schultz (1966), using acid phosphatase stain, correlated the antiviral activity of the macrophages with the state of lysosomal activation. Acid phosphatase staining revealed greater activation of lysosomes in vaccinia infected

mouse than in infected rabbit macrophages. Milligan (1983) found that the incubation of vaccinia virus with cell free lysates did not result in a drop in virus titer. He proposed that only the intact cell is responsible in some way for the expression of resistance against vaccinia virus infection.

Important to our understanding of the intracellular mechanism of innate resistance to vaccinia virus is knowing the exact point where viral replication is blocked. Work in this area, however, has produced conflicting results. Using electron microscopy and autoradiography, Ward found no evidence of first stage of uncoating and no apparent viral DNA synthesis. He suggested that vaccinia virus was degraded in the phagocytic vacuole. Along the same lines, Clark (1984) found that thioglycollate elicited mouse macrophages allowed the first stage of uncoating, but normal mouse macrophages did not.

Silverstein (1970), using electron microscopy and biochemical studies with ^{3}H-thymidine labeled IHD vaccinia virus observed the first stage of uncoating but not the second stage of uncoating. Kashanchi (1986), on the other hand, showed evidence of both the first and second stages of uncoating, but no viral DNA synthesis. He proposed that the block of the virus replicative cycle occurred at the narrow margin between the second stage of uncoating and viral DNA synthesis, and pointed to induction of interferon by mouse macrophages as the possible mechanism of resistance to vaccinia infection.

The purpose of this study was to follow the replication of intracellular vaccinia virus in both mouse and rabbit macrophages and to determine the point of viral inhibition in mouse macrophages. This information would allow the elucidation of the mechanism(s) which allow

normal mouse peritoneal macrophages to abort vaccinia infection and the determination of the fate of the virus. In particular, answers to the following questions were sought: Does the number of viral cores decrease with respect to time? This is important because it indicates the release of naked viral DNA and the occurrence of the second stage of uncoating. Is thymidine kinase produced in mouse macrophages? Because thymidine kinase is one of the earliest enzymes made, it can be used as a marker of early gene function. Radioactively labeled virus, biochemical tests and electron microscopy were used to gain answers to these questions.

MATERIALS AND METHODS

Experimental Animals

Young adult albino mice were obtained from the animal laboratory of Emporia State University, Emporia, Kansas. Young adult New Zealand white rabbits were obtained from breeders in the Emporia area.

Cell Culture Media

Mouse and rabbit macrophage harvesting medium contained Eagle's Minimum Essential Medium (MEM) consisting of l-glutamine, 2 units of heparin per ml, and combiotic (Penicillin G, 200 units per ml, and Streptomycin, 100 units per ml).

Cell culture media for both macrophages and fibroblasts contained MEM, combiotic, and consisted of 10 % sterile fetal calf serum (FCS). When necessary, the pH was adjusted using sterile 1.5 % or 7.5 % sodium bicarbonate.

A 5 % w/v stock solution of trypsin (Sigma, SP = 12,400 BAEE units 1 mg protein) was prepared in Dubelcco's phosphate buffer solution, without MG^{2+} or Ca^{2+} (D-PBS). It was membrane filtered and stored at $0^{o}C$. The final trypsinizing solution consisted of 0.25 % trypsin and 0.02 % EDTA.

Cell Culture Procedure

1. Mouse Fibroblast Cultures

Pregnant mice, 16 to 20 days gestation, were sacrificed by cervical dislocation, pinned onto a board, and sprayed with 70 % ethyl alcohol. The abdomen was opened by a longitudinal ventral incision of the skin made with scissors and forceps. The skin was

then cut away to expose the abdominal wall. The uterus was removed intact and placed in a beaker containing D-PBS with combiotic.

After rinsing, the uterus was opened, each embryo was removed separately and transferred to another petri dish containing D-PBS for washing. The embryos were minced using a sterile scalpel; the embryo fragments (about 1 cubic millimeter) were placed in 50 ml of MEM consisting of 0.25 % trypsin and 0.02 % EDTA. The cells were dispersed with a magnetic stirrer for 2 hours at room temperature.

At the end of this time 5 ml of sterile FCS were added to inactivate the trypsin and the cell suspension was filtered through a sterile cheese cloth. The filtrate was transferred to a centrifuge tube and washed twice with D-PBS at 2000 rpm for 10 minutes. The cells were counted using an improved Neubauer 6 hemacytometer and diluted to $2X10^6$ cells per ml with MEM consisting of 10 % FCS and combiotic. Fifty ml of the adjusted cell suspension were dispensed in Roux flasks and incubated at 37^oC for 36 h to 45 h (when the cells monolayered). The remaining cells 5 were diluted to 6X10 cells per ml with MEM containing 10 % FCS and combiotic and dispensed in 1 ml volumes in tissue culture flasks. The latter cell monolayers were used for virus titration.

2. Mouse Peritoneal Macrophages

Young adult mice were sacrificed by rapid dislocation of the neck, pinned onto a board and sprayed with 70 % ethanol. The outer skin was separated to expose the abdominal wall and resident macrophages were harvested by injecting 3 to 4 ml of MEM plus heparin into the peritoneum along the mid anterior line avoiding

puncturing the gut.

A needle was inserted into the flank and pulled sideways so as to form a pocket of fluid. The fluid was gently aspirated using a 10 ml syringe, transferred into cold centrifuge tubes and washed twice in cold D-PBS. After counting, the cells were adjusted to the appropriate concentration with MEM consisting of combiotic and 10 % FCS and dispensed in either flying coverslip tubes, cell culture flasks or milk bottles. The cells were allowed to settle for 2 h at 37^{o}C after which time the non adherent cells were washed away with warm D-PBS and fresh culture medium was added.

3. Rabbit Peritoneal Macrophages

Healthy young adult rabbits were injected intraperitoneally with 50 ml of sterile mineral oil (Revco heavy duty). Five days later, the animals were sacrificed by air embolism and abdominal hair was sprayed with 70 % ethanol. The skin layer was opened exposing the abdominal wall and 100 ml of MEM plus heparin and combiotic were injected into the peritoneum. The abdominal wall was gently massaged with sterile forceps for at least 1 min to suspend the macrophages.

The abdominal wall was opened, the cell suspension was removed with a sterile 100 ml volumetric pipette and emptied into a separatory funnel.

The funnel was held at 4 C for 10 min to allow separation of the aqueous and oil phases. The aqueous phase, containing the cells, was transferred into centrifuge tubes, centrifuged at 2000 rpm for 10 min, and washed twice with fresh D-PBS.

The cells were counted using a Neubauer hemacytometer, diluted to the appropriate concentration, and dispensed in either flying coverslip tubes, cell culture flasks, or milk bottles. After incubating for 2 h at 37°C, the non-adherent cells were washed away with warm D-PBS. Fresh MEM plus combiotic and 10 % FCS was replaced.

Staining of Cells on Coverslips

Coverslips were washed with D-PBS, allowed to air dry and fixed for 5 min in absolute methanol. They were stained for 9 min with May Grunwald stain and for 14 min with Giemsa stain. After staining, the coverslips were rinsed in acetone followed by a 1:1 acetone-xylene mixture, cleaned in xylene, and mounted cell side down on a glass slide using a Kleermount-Xylene solution.

Vaccinia Virus

1. Virus Propagation

The IHD strain of vaccinia virus was originally obtained from the American Type Culture Collection. It was propagated on mouse fibroblast monolayer cultures.

Roux flasks containing fibroblast monolayers were washed twice with D-PBS and inoculated with 10 ml of MEM containing 2 % FCS and 20 plaque forming units (PFU) of IHD vaccinia per ml, and incubated at 37°C for 2 h with frequent agitation. After adsorption, the inoculum was poured off and the fibroblast monolayers were washed with warm D-PBS. Fifty ml of fresh MEM containing 10 % FCS were replaced and the cells were incubated until extensive cellular

destruction was observed.

Radioactively labeled IHD vaccinia virus was prepared by incubating infected mouse embryo monolayers in 60 ml of MEM 3 containing ^{3}H thymidine (ICN Radiochemicals) at a final concentration of 10 uc per ml. Again, incubation was continued until such time as extensive viral-induced cell damage was observed.

2. Harvesting of Virus

Roux flasks were subjected to 3 cycles of freeze-thaw. The viral suspension was placed in centrifuge tubes and centrifuged at 200 rpm for 10 min to remove cell debris. The supernatant was pooled and stored at -70°C until needed.

3. Virus assay

Ten-fold dilutions of the virus sample were made using MEM consisting of 2 % FCS. One ml of virus dilution was added per culture flask containing almost monolayered mouse embryo fibroblasts and incubated for 2 h at 37°C with frequent agitation. The inoculum was removed and the fibroblasts were washed with warm D-PBS. Two ml of fresh MEM plus combiotic were replaced. The flasks were incubated at 37°C for 36 to 48 h when plaque formation was evident.

The medium was poured off and the monolayers were stained with Gram's crystal violet diluted 1:10 with water. The stain supernatant was removed, the monolayers were washed twice with tap water, and plaque were counted. The virus titer was calculated and the viral activity was expressed as (PFU) per ml.

4. Purification of labeled virus

After viral multiplication, the cell monolayers containing radioactively labeled IHD vaccinia virus were subjected to 3 cycles of freeze-thaw followed by low speed (2000 rpm for 10 min) centrifugation to remove cell debris. The supernatant was pooled, layered on 5 ml of 36 % sucrose (w/v) in distilled water, and centrifuged at 25000 rpm for 100 minutes.

The pellet contained both intact virus and cellular DNA. Cellular DNA and exposed viral DNA were eliminated by resuspending the pellet in 5 ml of D-PBS containing DNase I (200 ug/ml) in 50 mM Mg^{2+} for 30 min at 37°C. Viral clumps were dispersed by sonification (0.6 amps for 5 cycles of 15 sec). The resulting solutions were layered on 5 ml of 36 % sucrose and centrifuged at 25000 rpm for 100 minutes. The viral-containing pellets were resuspended in 10 ml of MEM plus combiotic, 0.1 ml of which was used to determine the virus titer.

Electron Microscopy

Preparation of Peritoneal Macrophages

After harvest the cells were diluted, and adjusted to 1 X 10^9 cells per ml. Five ml volumes of this suspension were dispersed in glass milk bottles and incubated at 37 C for 2 hours. Non-adherent cells were removed by two washes in D-PBS and 10 ml of fresh Earle MEM plus combiotic supplemented with 10 % FCS were added. The cells were incubated for an additional 2 hours.

Cell Culture Infection

In order to achieve a high multiplicity of infection (MOI) for electron microscopy work, virus was first concentrated by centrifugation for 90 min at 4°C at 25000 rpm using a fixed angle head. The virus was resuspended in culture medium to give a titer of $6X10^7$ PFU per ml. Viral clumps were dispersed by sonification in a Raytheon sonic oscillator at 0.6 amperes for 5 cycles of 15 sec each.

The macrophage cultures were cooled for 5 min at 4°C, washed twice with cold D-PBS, infected with 5 ml of IHD vaccinia virus (also precooled), and incubated at 4°C for 1 h with frequent agitation. The inoculum was poured off, the macrophage cultures were washed 3 times with 5 ml of cold D-PBS, and incubated at 37°C with 10 ml of fresh medium for 0, 3, 6, or 12 hours. Five ml of $3X10^6$ PFU per ml were used to infect macrophage cultures that were incubated for 24 hours. Time zero was the time when the infected macrophage cultures reached 37°C.

Cell Processing

Infected macrophages for examination by transmission electron microscopy were cooled at 4°C for 5 min, scraped with a rubber policeman from the surface of the glass, transferred into conical tubes and washed 3 times for 15 min each using cold D-PBS. Centrifugation was carried out in the cold (6°C) at 1800 rpm for 7 min.

Pre-cooled solutions of glutaraldehyde and osmium tetroxide 2:1 were combined at 4°C immediately prior to use. The resultant mixture was used to fix the cells for 75 min at 4°C, (Jones and Hirsch 1972) followed by 3.15 min washings with cold D-PBS.

The cells were stained at room temperature for 30 min using 0.2 %

uranyl acetate in 70 % ethanol, followed by 3-15 min washings with warm D-PBS. Warm, melted Noble agar was added to the cell pellet and 1 cubic mm blocks were cut where clumps of cells were apparent. These blocks were dehydrated successively for 10 min in 60 %, 80 % and 95 % ethanol, and 3 times for 10 min each in 100 % ethanol.

The cells were then washed 3 times for 10 min each in propylene oxide. After the third wash, a working mixture of propylene oxide plus epon araldite -DMP30 (1:1 v/v) was added. An hour later, an equal volume of working mixture was added and left for 8 hours.

The tissue was placed in plastic Beem capsules filled with pure working mixture. After embedding, the blocks were cured by an overnight incubation in a 35°C oven, followed by 12 h incubation at 45°C, and then 24 h incubation at 60°C.

The blocks were removed from the capsules, trimmed and sectioned using an LKB ultramicrotome. The sections were picked up using 300 mesh copper grids, allowed to air dry and stained with 2 % aqueous uranyl acetate for 20 min followed by 5 min of Reynold's lead citrate which had been filtered 24 h prior to use using a 0.01 micron membrane filter to remove any precipitate. After staining the sections were allowed to air dry. They were then examined and photographed using a Hitachi Electron Microscope (Model HS-8).

Film processing

Photographic negatives were developed in D-19 developer for 4 min, rinsed with tap water for 2 min, fixed for 10 min and rinsed with tap water for 30 minutes. After allowing to air dry overnight, the resulting negatives were enlarged and developed on Polycontrast Rapid II RC Kodak paper.

Biochemical Studies

1. Assay of Second Stage Uncoating

Macrophage monolayers in culture flasks were cooled for 5 min at 4°C, inoculated with 1 ml of ^{3}H DNA labeled virus at MOI of 10 and incubated at 4°C for 30 min with frequent agitation. The inoculum was poured off, the macrophage cultures were washed with cold D-PBS, and then warmed at 37°C to initiate a synchronous wave of viral penetration. At 30 min, 4 h and 8 h after warming, the cells were scraped from the surface of the culture flasks with a rubber policeman, transferred into small test tubes and centrifuged at 2000 rpm for 10 min to deposit the cells.

The cell pellet was suspended in 2.2 ml of a solution consisting of 0.01 M sodium phosphate and subjected to two cycles of freeze-thaw. At this point, 0.2 ml quantities from each sample, representing the total count, were counted on an LKB liquid scintillation counter.

Half of the remaining sample was treated with trichloroacetic acid (TCA) (0.2 ml of 50 % stock TCA); the other half was treated with TCA plus 500 ug/ml DNase I. Both halves were incubated at 37°C for 30 minutes. The samples were then held overnight at 4°C and centrifuged at 2500 rpm for 5 minutes. Radioactivity of the supernatant and pellet were counted.

2. Thymidine Kinase Activity

Thymidine kinase activity was assayed essentially as described by Bollum and Potter (1959). Macrophage monolayers in culture flasks were at MOI of 1.0 and incubated at 4°C for 1 h with

frequent agitation. The inoculum was poured off, the macrophage cultures were washed with cold D-PBS, 5 ml of cold fresh culture medium was replaced and the infected cells were warmed to 37°C. At 0, 3, 6, 12, and 24 h after warming, the cells were scraped off the culture flasks using a rubber policeman, transferred into small test tubes and centrifuged for 10 min at 2000 rpm.

The cell pellet was suspended in 1 ml of Tris-HCl buffer at pH 8.0, subjected to 3 cycles of freeze thaw and 3.15 sec cycles of sonification. The resulting lysate was centrifuged at 2500 rpm for 10 min to remove cell debris.

Forty ul of the supernatant solution from the above step and 100 ul of a substrate mixture consisting of 0.2 M-tris buffer, pH 8.0, were incubated for 20 min at 37°C. The substrate mixture provided the following substances and concentrations: ATP 5mM; Mg^{2+}, 5 mM; and ^{3}H thymidine 0.2 mci per ml. The reactions was terminated by the addition of 20 ul of 50 % ethylenediamine-tetraacetic acid (EDTA).

Fifty ul of the above reaction mixture was spotted onto a piece of ion exchange paper (2.3 cm Whatman DE 81). The paper was rinsed twice in ammonium formate, once with distilled water and once with absolute ethanol. Each of the paper circles was transferred to a glass vial and stored for 5 h at room temperature. Five ml Scinti Verse II (Fisher Scientific Company) were then added. The samples were shaken vigorously for 1 min and counted for 20 min periods using a liquid scintillation counter. A zero enzyme sample consisting of D-PBS and uninfected macrophage enzyme extract were used as controls.

RESULTS

Normal mouse peritoneal macrophages, planted on coverslips and observed after staining with May Grunwald and Giemsa stains, revealed round cells with a bean shaped to round nucleus (Fig. 1). Elicited rabbit peritoneal macrophages also appeared rounded, but they were moderately vacuolated (fig. 2).

Mouse and rabbit macrophage cultures infected with vaccinia virus at MOI of 1.0 were stained at time intervals with May Grunwald-Giemsa stain and examined with the light microscope. By 72 h postinfection, rabbit macrophages (Fig. 3) showed typical cytopathogenic effects, marked by cell rounding and aggregation. There were characteristic nuclear changes, with many nuclei showing fragmentation and pyknosis.

In contrast, infected mouse macrophages (Fig. 4) showed no evidence of cytopathogenic changes at 72 h postinfection, thus substantiating the ability of normal mouse macrophages to inhibit vaccinia virus replication and to escape its deleterious effects. Mouse macrophages tended to retain their characteristic morphology and spread on the glass surface.

Ultrastructure study of these macrophages revealed cells containing typical cellular organelles. Rabbit macrophages tended to be more vacuolated (Fig. 5) and mouse macrophages contained a significantly greater number of small vescicles (Fig. 6).

Fig. 1. Mouse peritoneal macrophages (X 100)
May Grunwald-Giemsa stain.

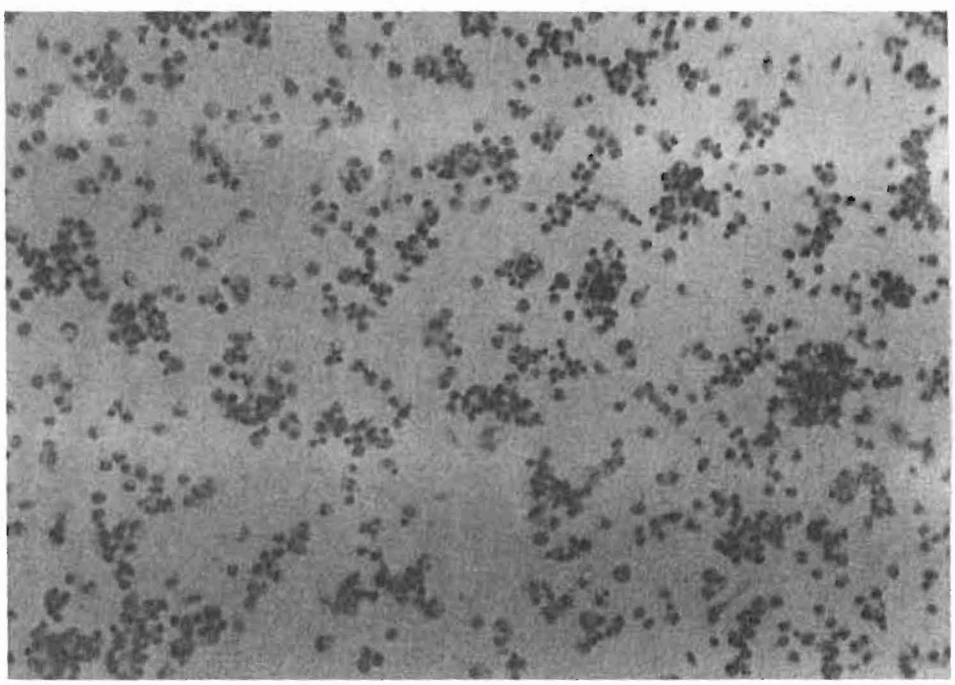


Fig. 2. Rabbit peritoneal macrophages (X 100)
May Grunwald-Giemsa stain.

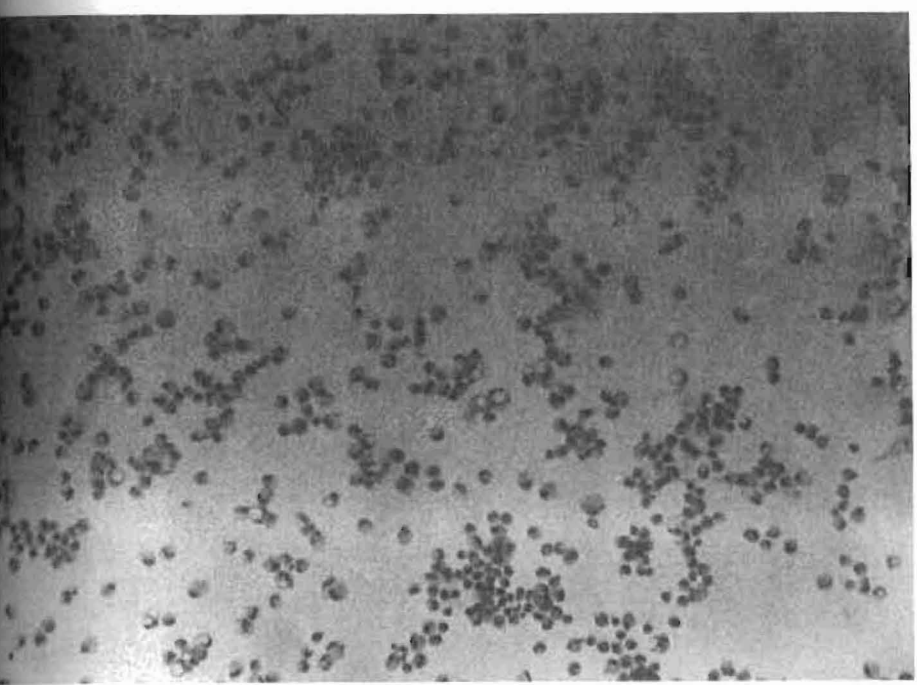
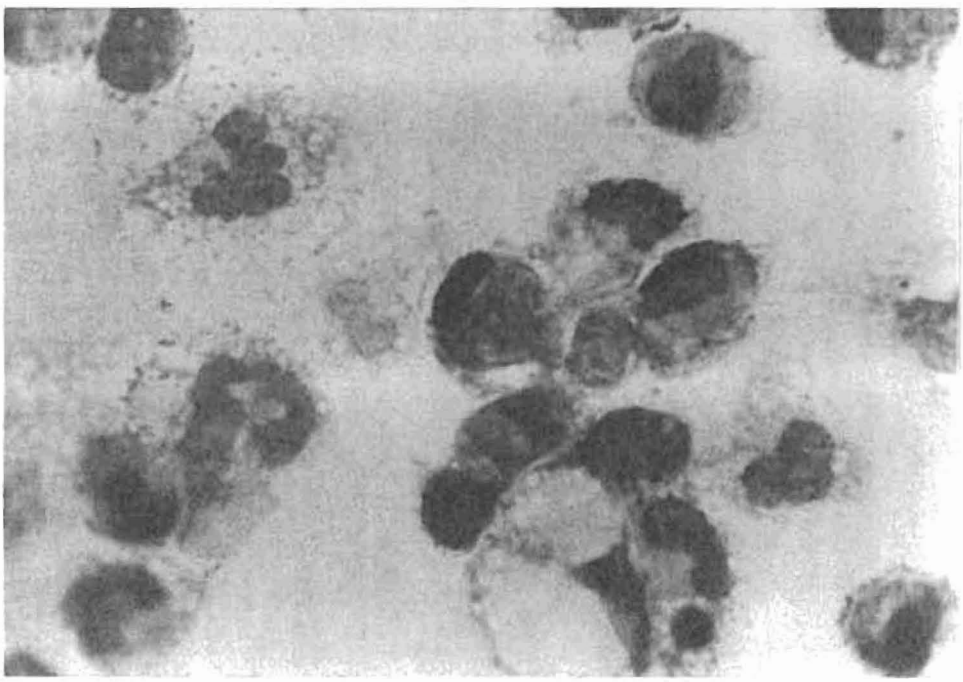


Fig. 3. Infected rabbit peritoneal macrophages at MOI of 1.0 after 72 h incubation (X 400) May Grunwald-Giemsa stain.



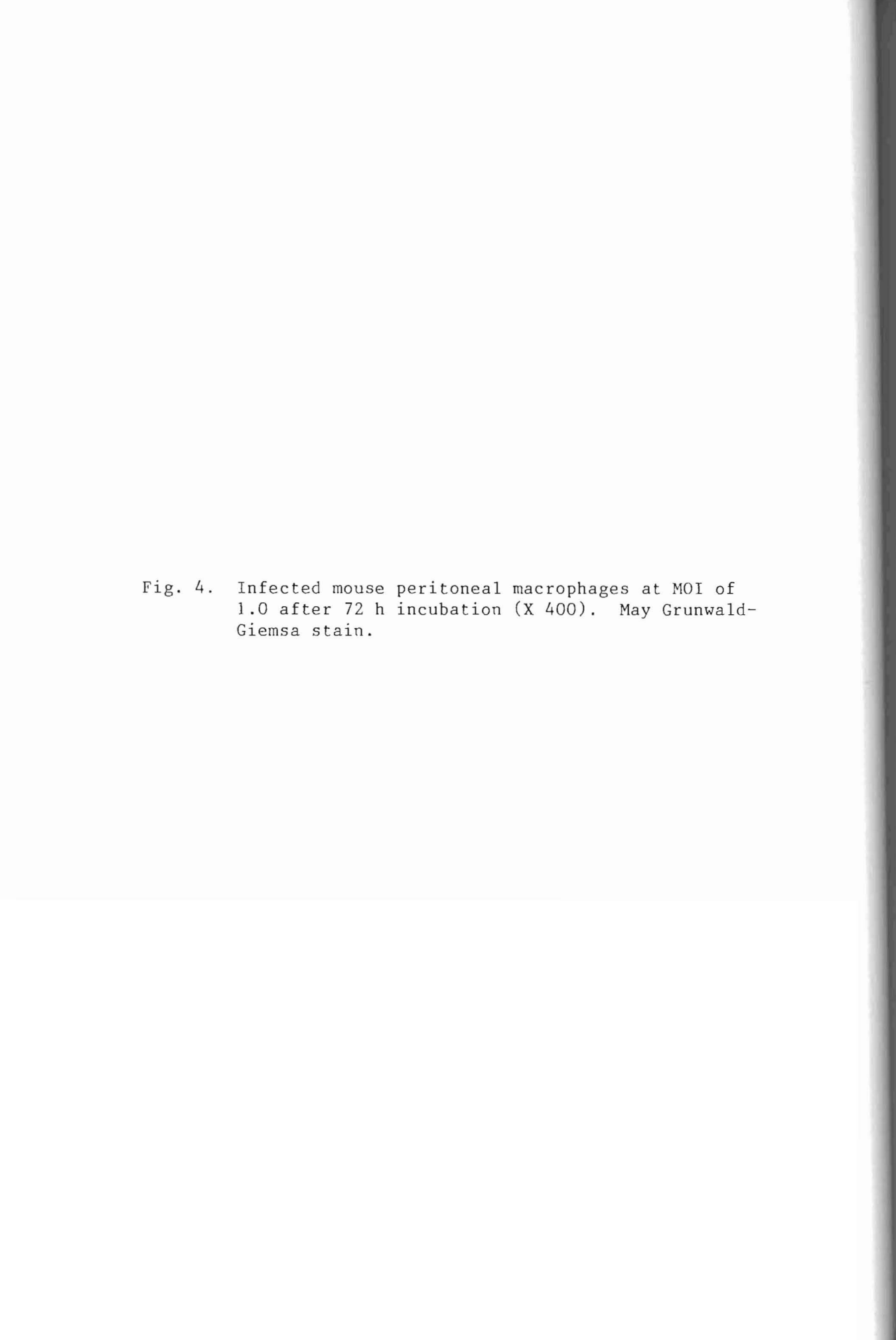


Fig. 4. Infected mouse peritoneal macrophages at MOI of 1.0 after 72 h incubation (X 400). May Grunwald-Giemsa stain.

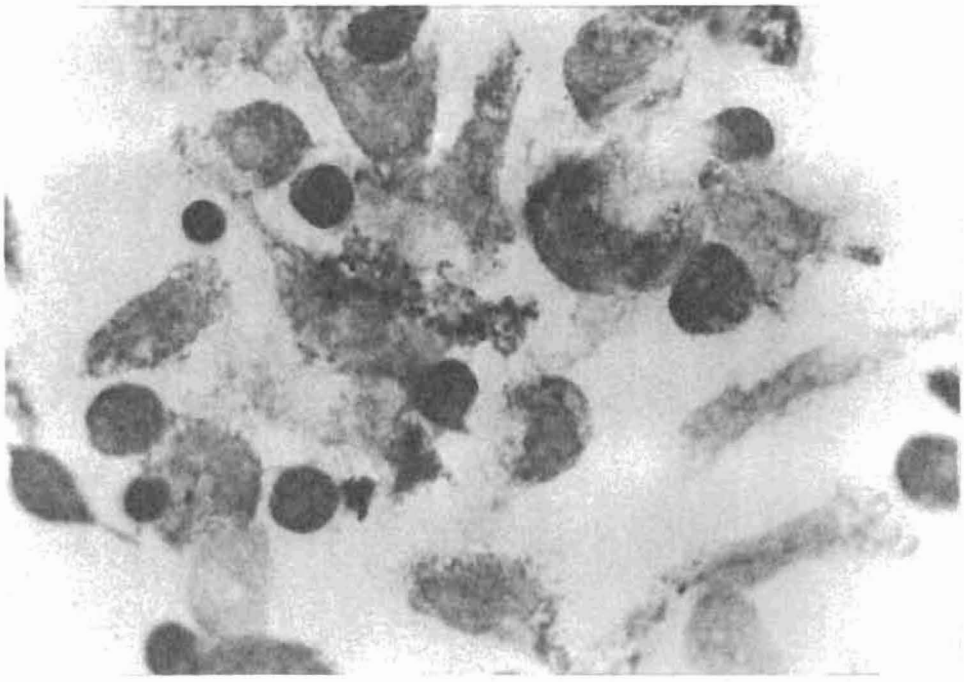
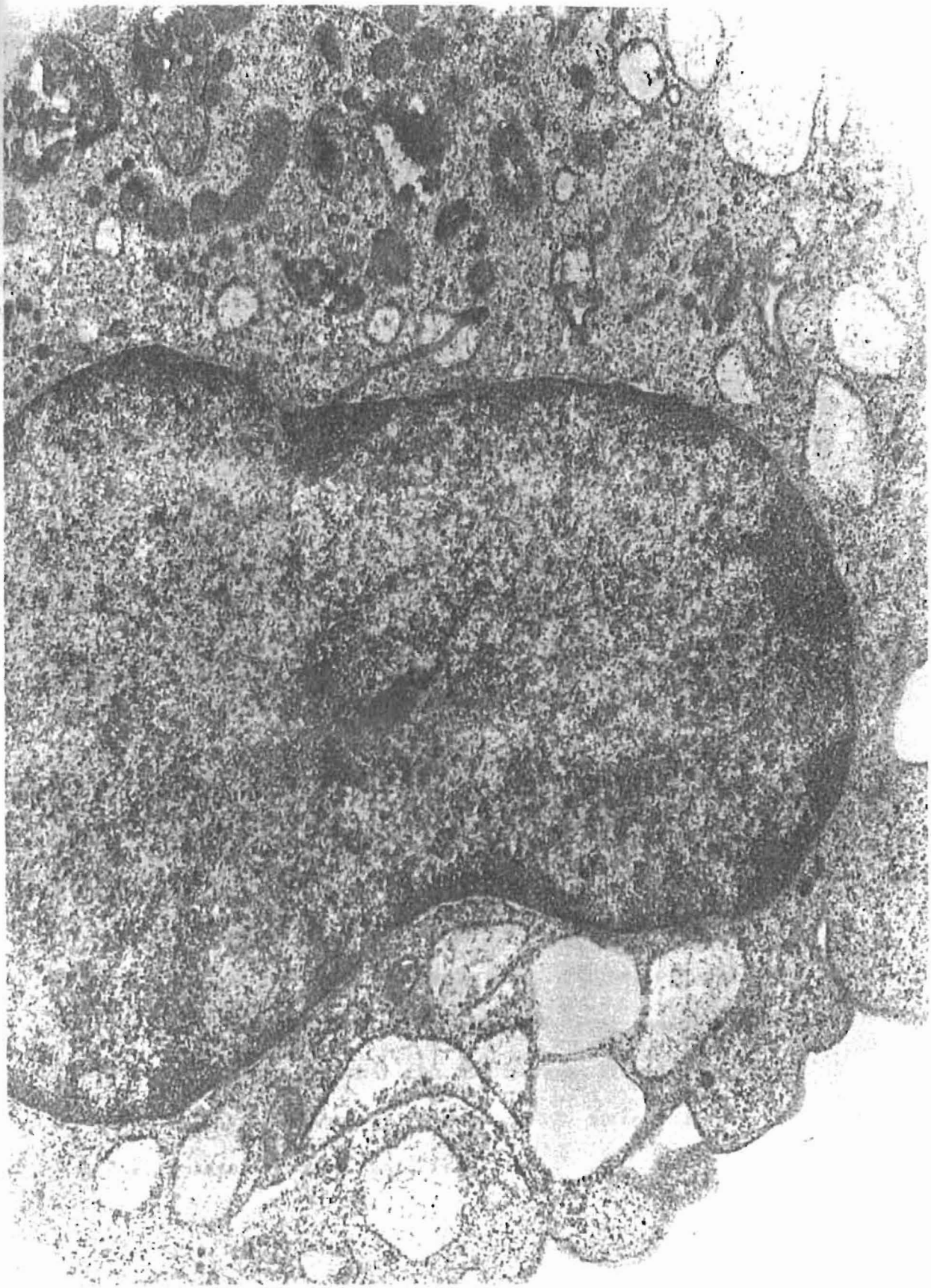


Fig. 5. Electron micrograph of a rabbit peritoneal macrophage after 8 h incubation. Several mitochondria and cytoplasmic vesicles of varying sizes are seen. (X 38,000) Uranyl acetate/lead citrate stain

Fig. 6. Electron micrograph of a mouse peritoneal macrophage after 10 h incubation. Cytoplasmic vesicles and phagocytic vacuoles are seen. (X 38,000) Uranyl acetate/lead citrate stain.



Electron Microscopy

Macrophages infected with vaccinia virus at MOI of 20 were processed for electron microscopy at 0, 3, 6, 12, and 24 h after the initial adsorption. Adsorption was carried out at 4 c for 60 min, and the cells were warmed to 37 c to initiate a synchronous wave of phagocytosis. To insure that the same cell was not examinee twice, five blocks were sectioned per time post adsorption, and the blocks were trimmed with a razor blade after sectioning.

1. Attachment

   Zero h samples revealed adsorption of vaccinia virus to mouse macrophages (Figs. 7, 8) and to rabbit macrophages (Fig. 9).

2. Viral Phagocytosys

   Sections of infected mouse macrophages (Fig. 8) and infected rabbit macrophages (Fig. 10) contained typical viral particles within cytoplasmic vacuoles. Frequently, a single virus-containing vacuole was observed per cell, but a significant number of sections were found containing two or more phagocytic vacuoles surrounding viral particles.

3. First stage of uncoating

   Three h samples showed viral cores in the cytoplasms of both rabbit (Figs. 10, 11) and mouse (Fig. 12) macrophages. The presence of viral cores indicated that the virus was not destroyed by lysosomal enzymes and that the first stage of uncoating had occured. At 6 h postinfection, some rabbit macrophages also showed cytoplasmic inclusions (Fig. 13).

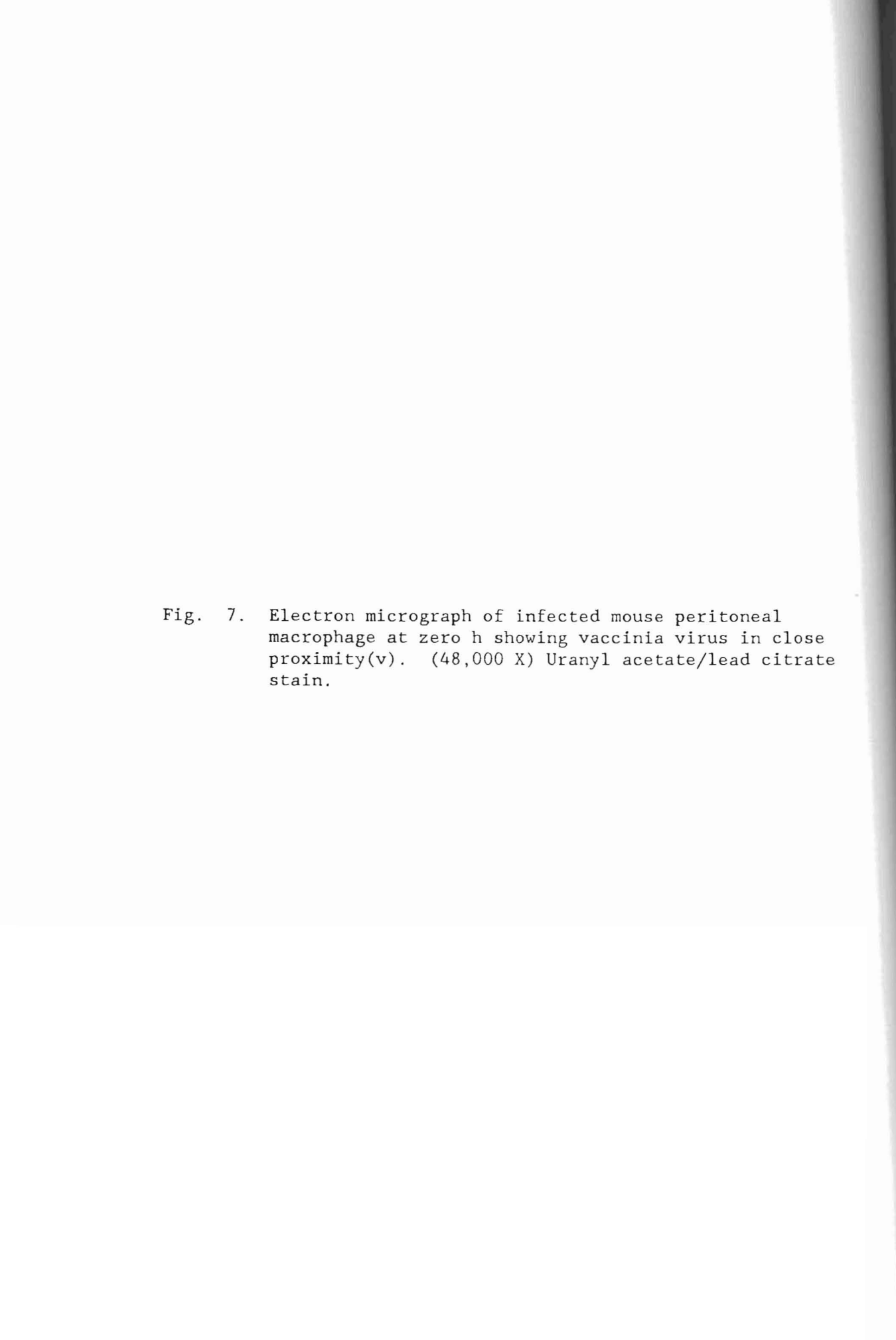


Fig. 7. Electron micrograph of infected mouse peritoneal macrophage at zero h showing vaccinia virus in close proximity(v). (48,000 X) Uranyl acetate/lead citrate stain.

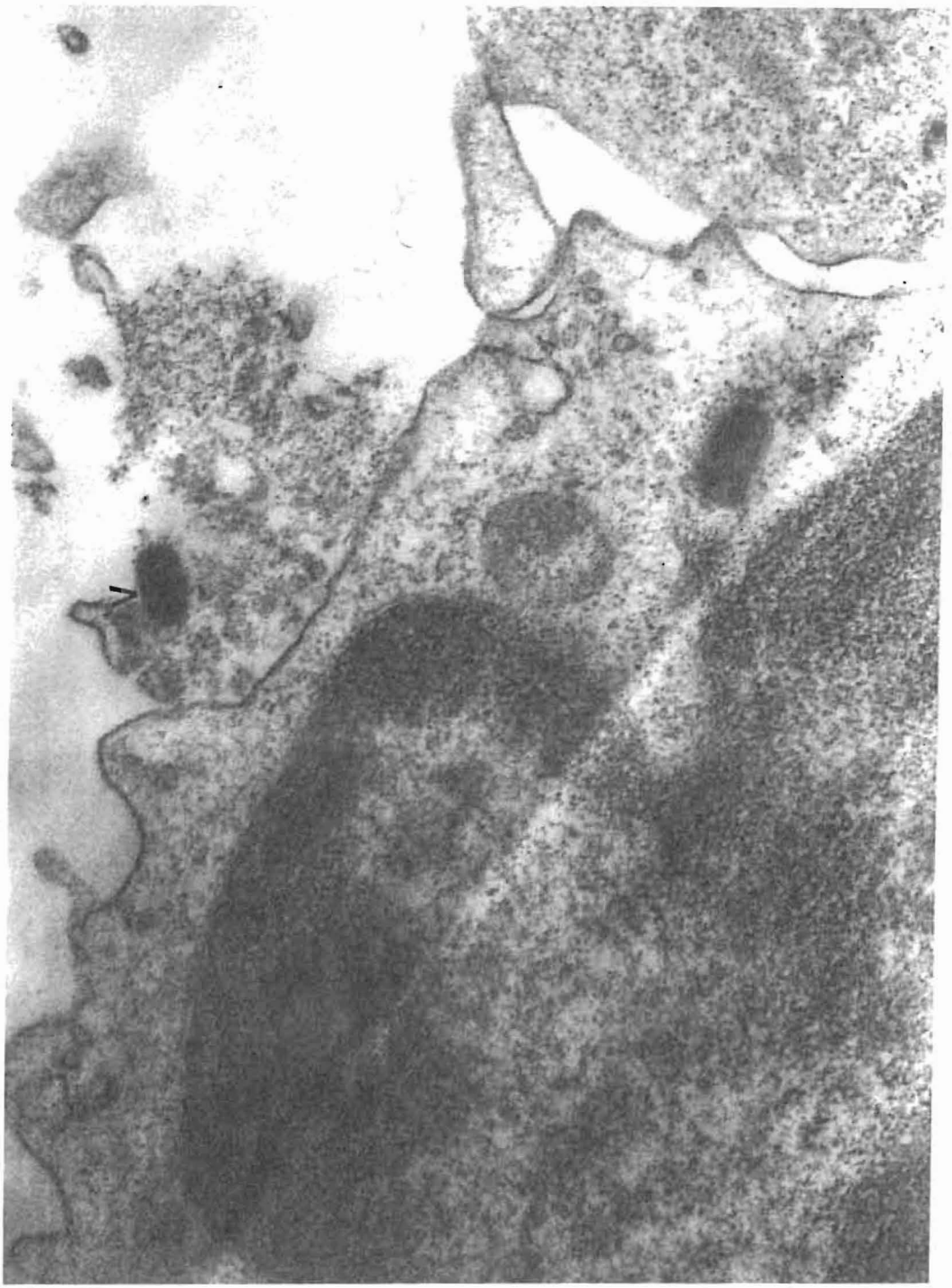
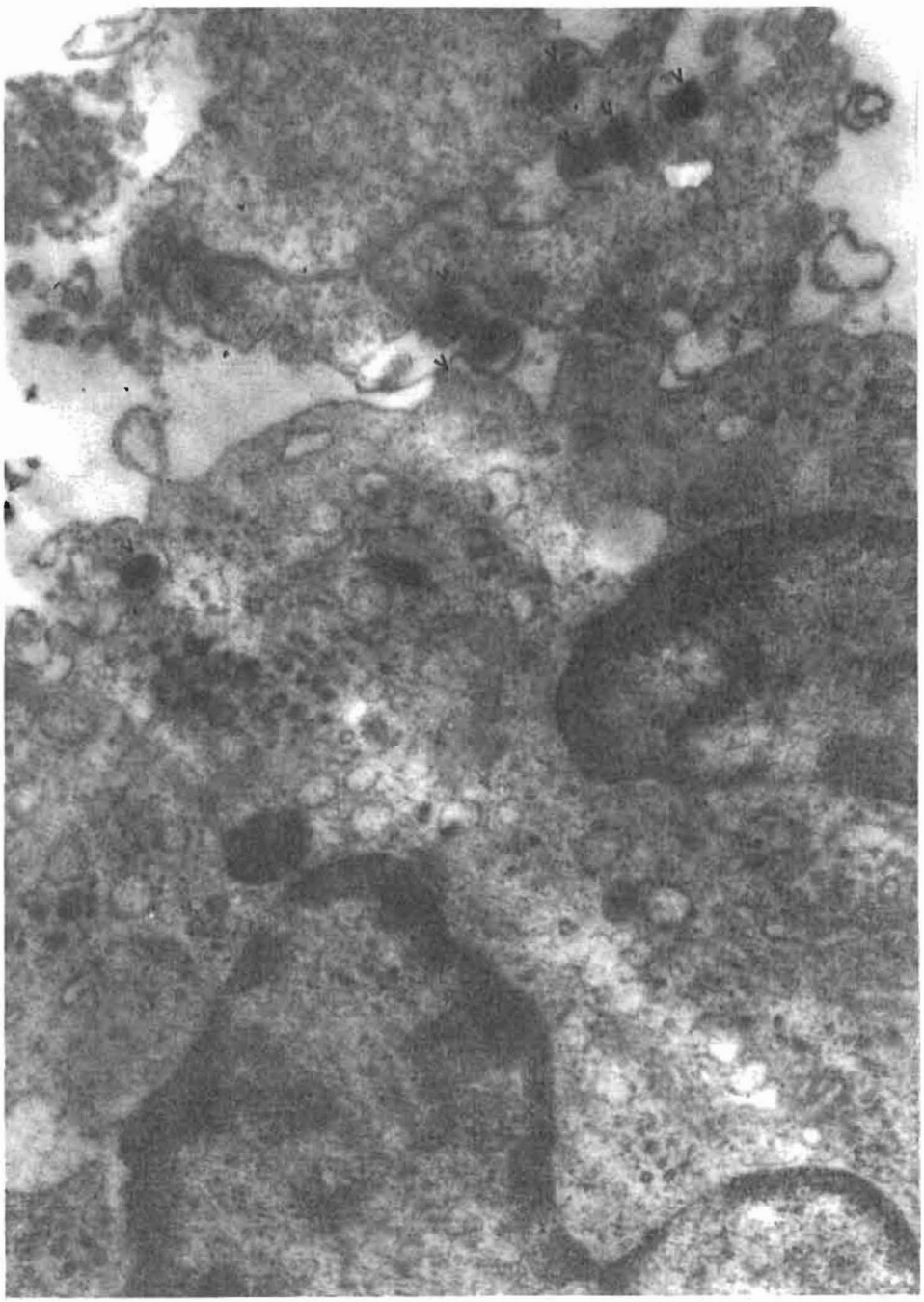


Fig. 8. Electron micrograph of infected mouse macrophages at zero h showing attachment and phagocytosis of vaccinia virus (v). (X 36,000) Uranyl acetate/lead citrate stain.



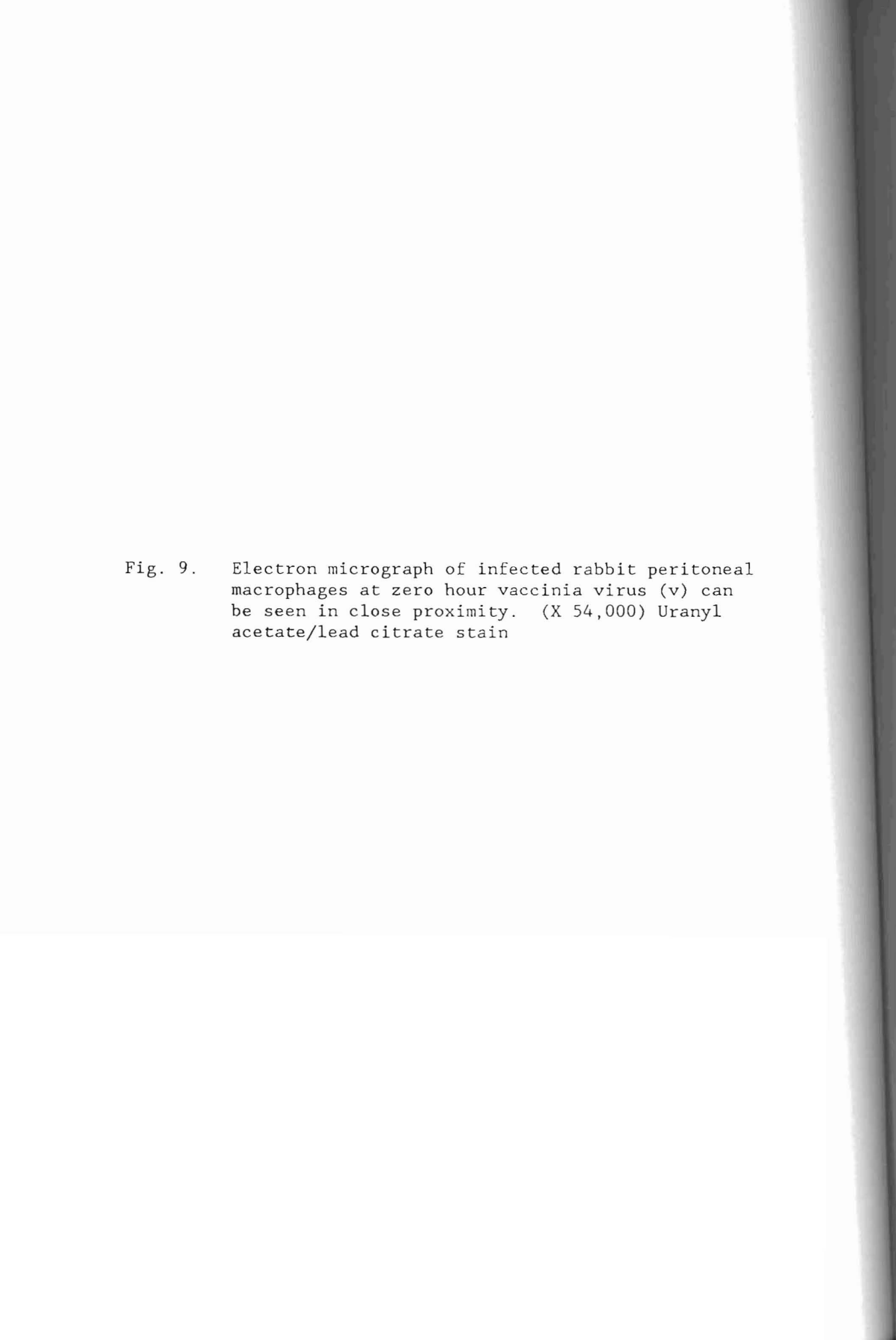


Fig. 9. Electron micrograph of infected rabbit peritoneal macrophages at zero hour vaccinia virus (v) can be seen in close proximity. (X 54,000) Uranyl acetate/lead citrate stain

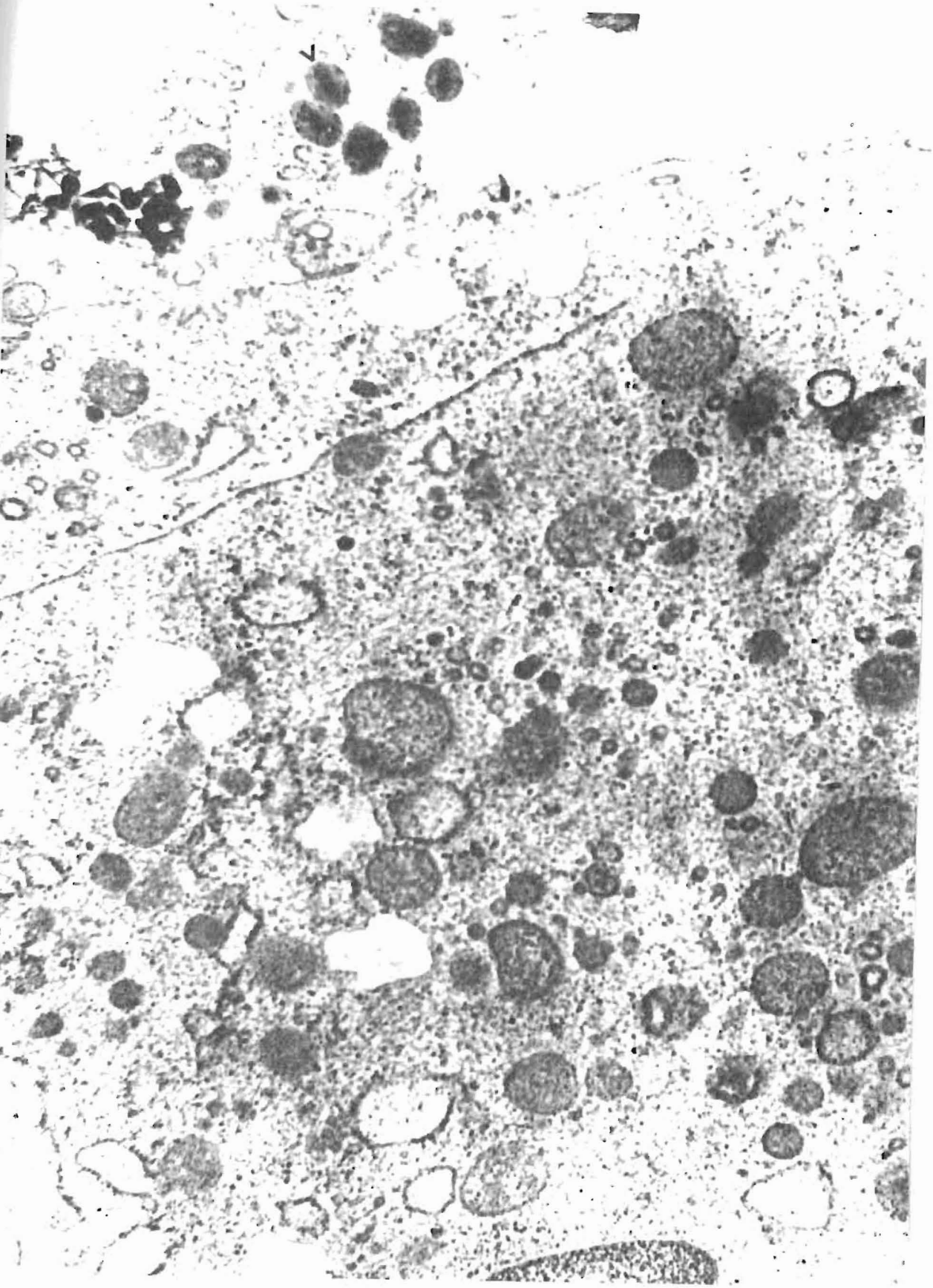


Fig. 10. Electron micrograph of infected rabbit macrophage after 3 h showing phagocytosis and 3 viral cores in the cytoplasm (v). (X 54,000) Uranyl acetate/lead citrate stain.

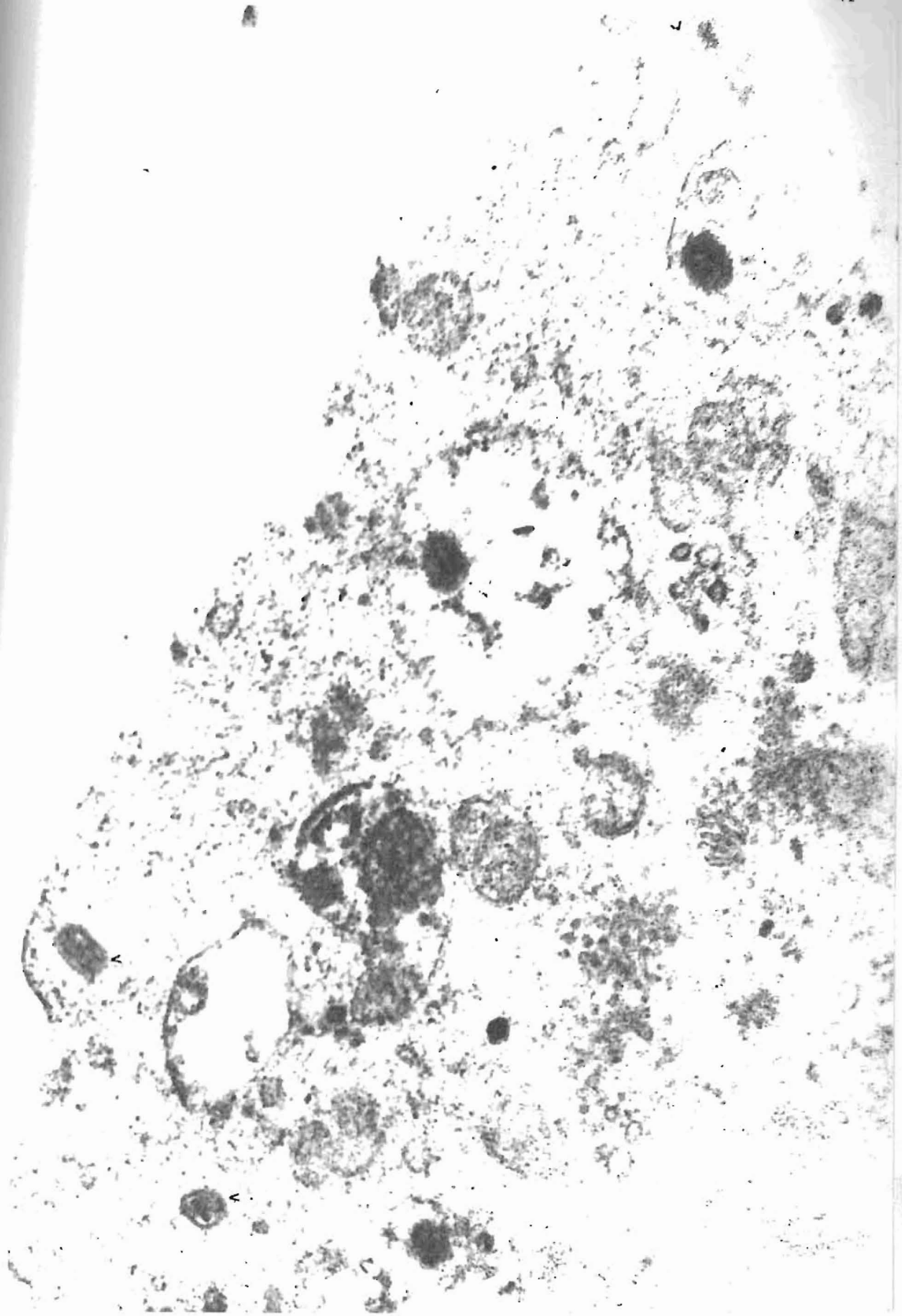


Fig. 11. Electron micrograph of infected rabbit macrophage after 3 h showing a viral core in the cytoplasm (v). (X 54,000) Uranyl acetate/lead citrate stain.

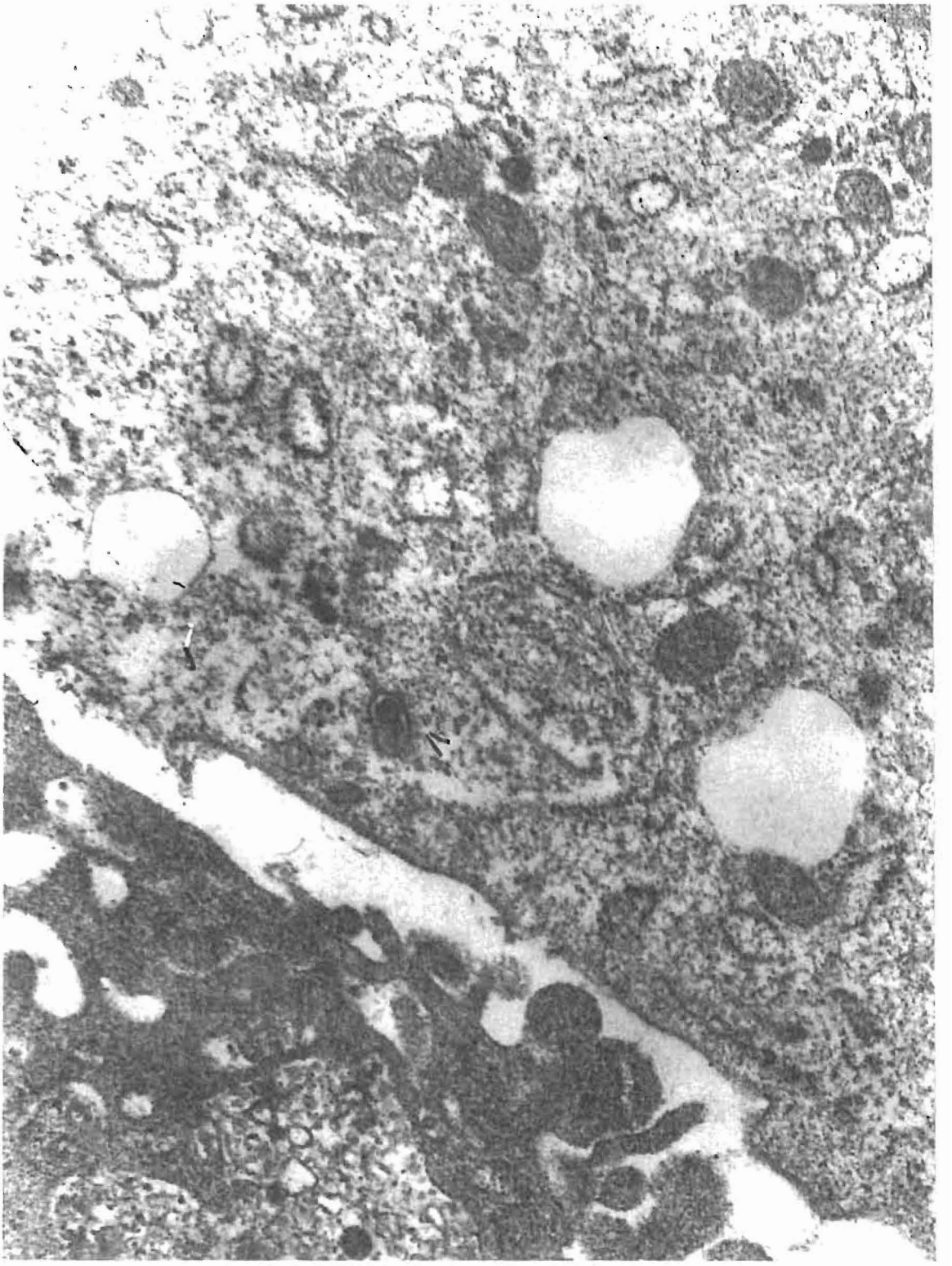


Fig. 12. Electron micrograph of infected mouse peritoneal macrophage. A viral core and its spikes can be seen (v). (X 54,000) Uranyl acetate/lead citrate stain.

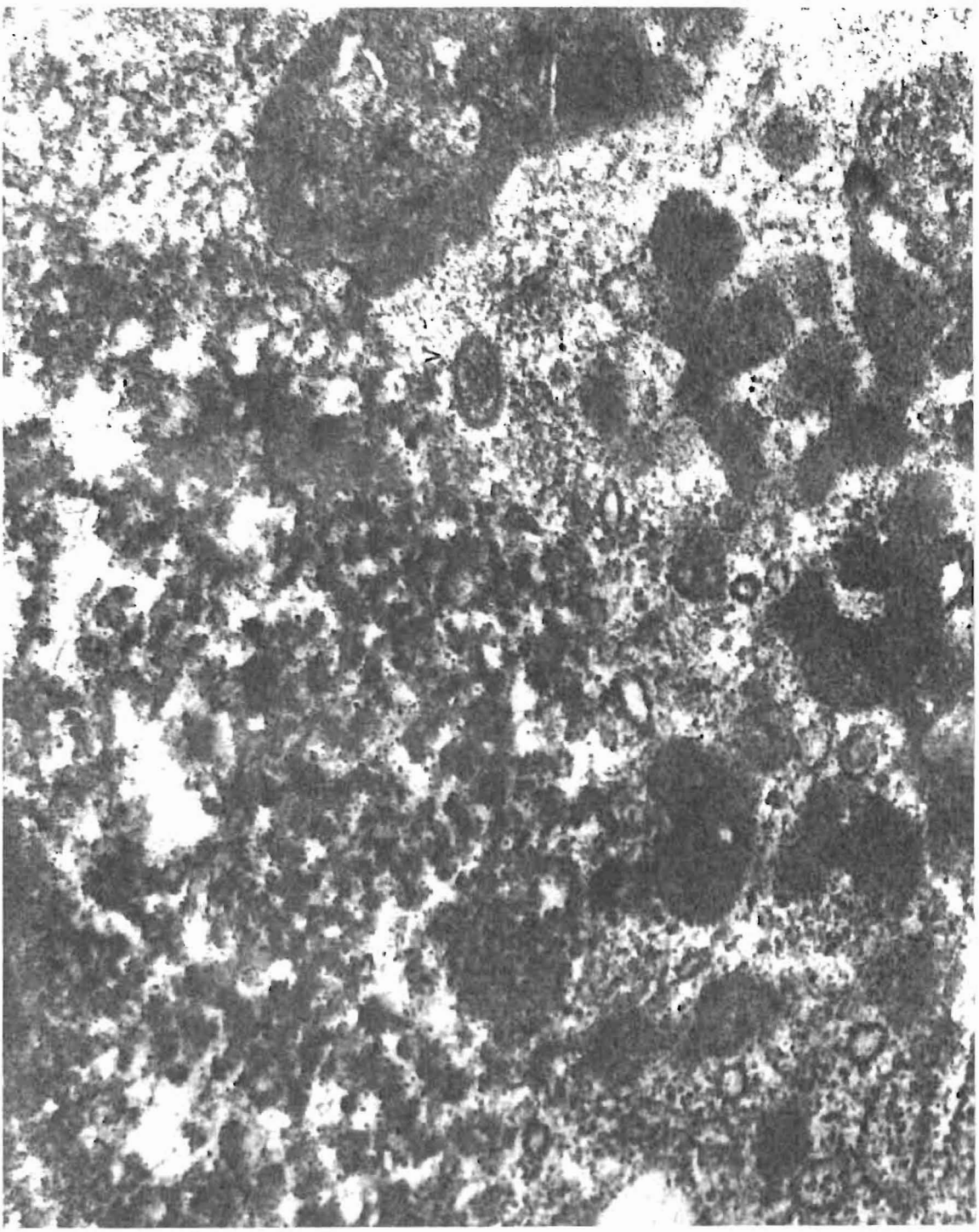
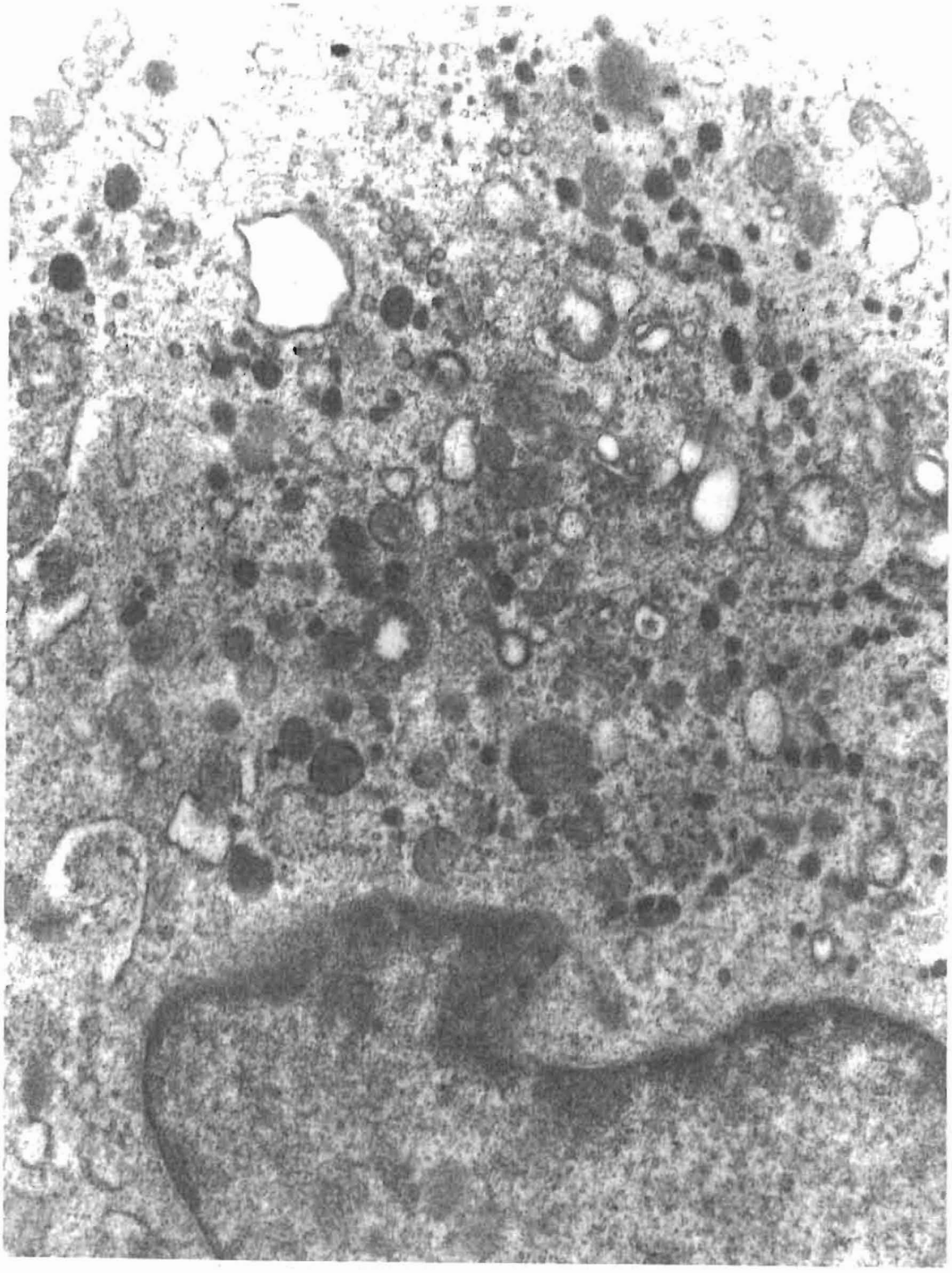


Fig. 13. Electron micrograph of infected rabbit peritoneal macrophage after 6 h showing cytoplasmic inclusions. (X 48,000) Uranyl acetate/lead citrate stain.



One hundred and fifty sections at 0, 2, 3, and 6 h postinfection were counted to obtain a quantitative comparison between mouse and rabbit macrophages relative to the first stage of uncoating (Table 1). No cores were observed in either system at 0 h postinfection. At 3 h, approximately 47 % of mouse macrophages and 49 % of rabbit macrophages contained viral cores. The accumulation of cores in the cytoplasms provided additional evidence for the occurrence of the first stage of uncoating in both systems. At 3 h postinfection, 16 % of rabbit macrophages and 13 % of mouse macrophages contained viral cores. At 6 h post infection, 3 % of rabbit macrophages contained cores and no cores were observed in mouse macrophages. The decrease in the number of cores from 2 to 6 h post infection suggested the breakdown of cores, characteristic of the second stage of uncoating.

4. Viral assembly

Electron micrographs of late infection revealed immature forms of vaccinia virus in rabbit macrophages (Figs. 15, 16, 17, 19). In contrast, no evidence of viral replication was observed in mouse macrophages (Figs. 14, 18, 20), confirming that viral inhibition occurred at an earlier stage in the replication cycle.

For quantitative comparison, 100 sections were examined for viral factories at 12 and 24 h post infection (Table 2). No viral factories were observed in mouse macrophages at either time period whereas 23 % and 35 % of rabbit macrophages contained viral factories at 12 and 24 h, respectively.

Table 1. Number of vaccinia virus cores observed in 150 sections of macrophages with respect to ttime.

Table 1. Number of viral cores per 150 sections of mouse and rabbit macrophages with respect to time.

EVIDENCE OF FIRST STAGE UNCOATING		
Time	Number of Cores	
	Rabbit	Mouse
0 hrs	0	0
2 hrs	71	73
3 hrs	24	19
6 hrs	5	0

Fig. 14. Electron micrograph of infected mouse peritoneal macrophage after 6 h (X 46,000) Uranyl acetate/lead citrate stain.

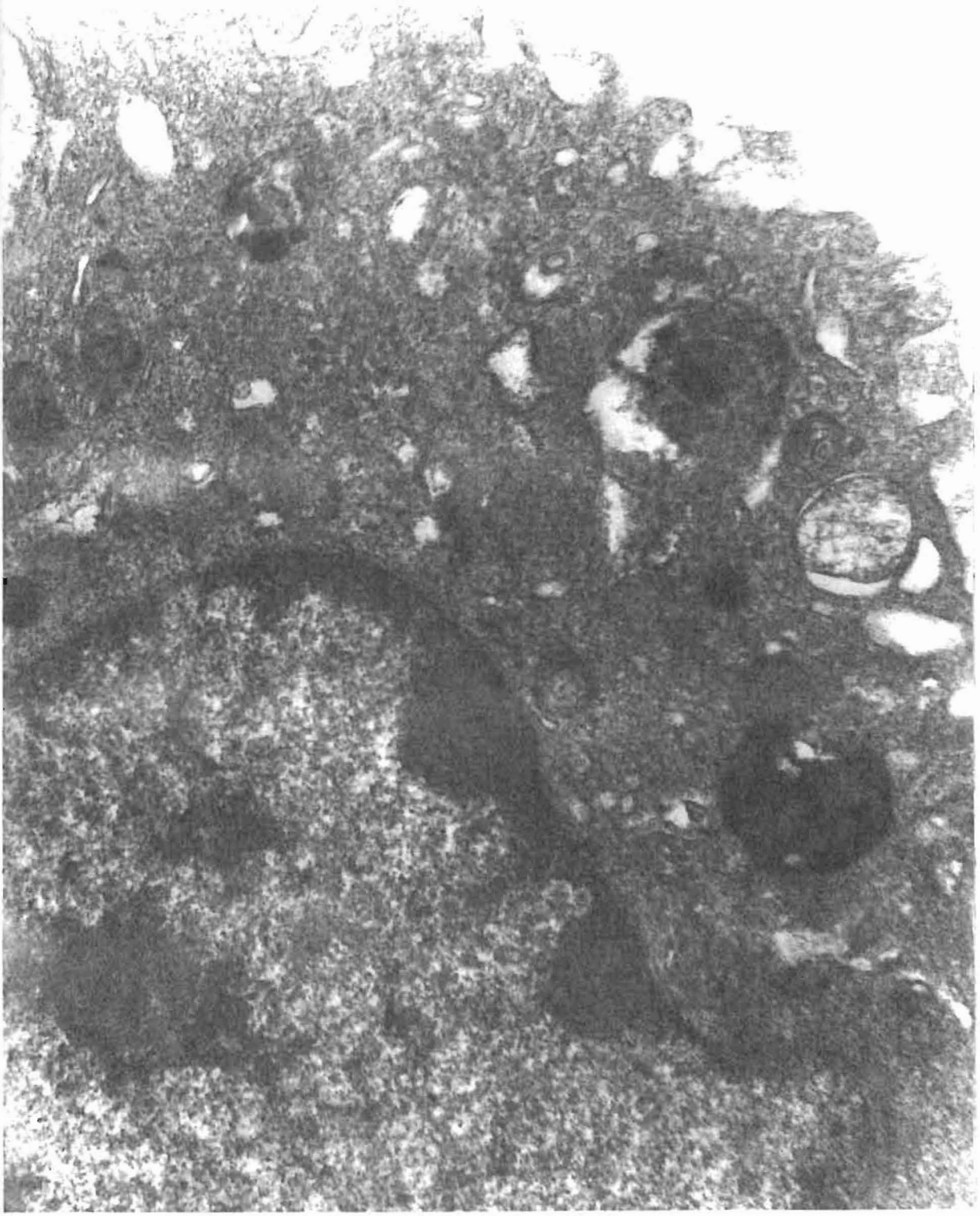


Fig. 15. Infected rabbit peritoneal macrophage after 12 h incubation. Immature virus can be seen (v). (X 54,000) Uranyl acetate/lead citrate stain

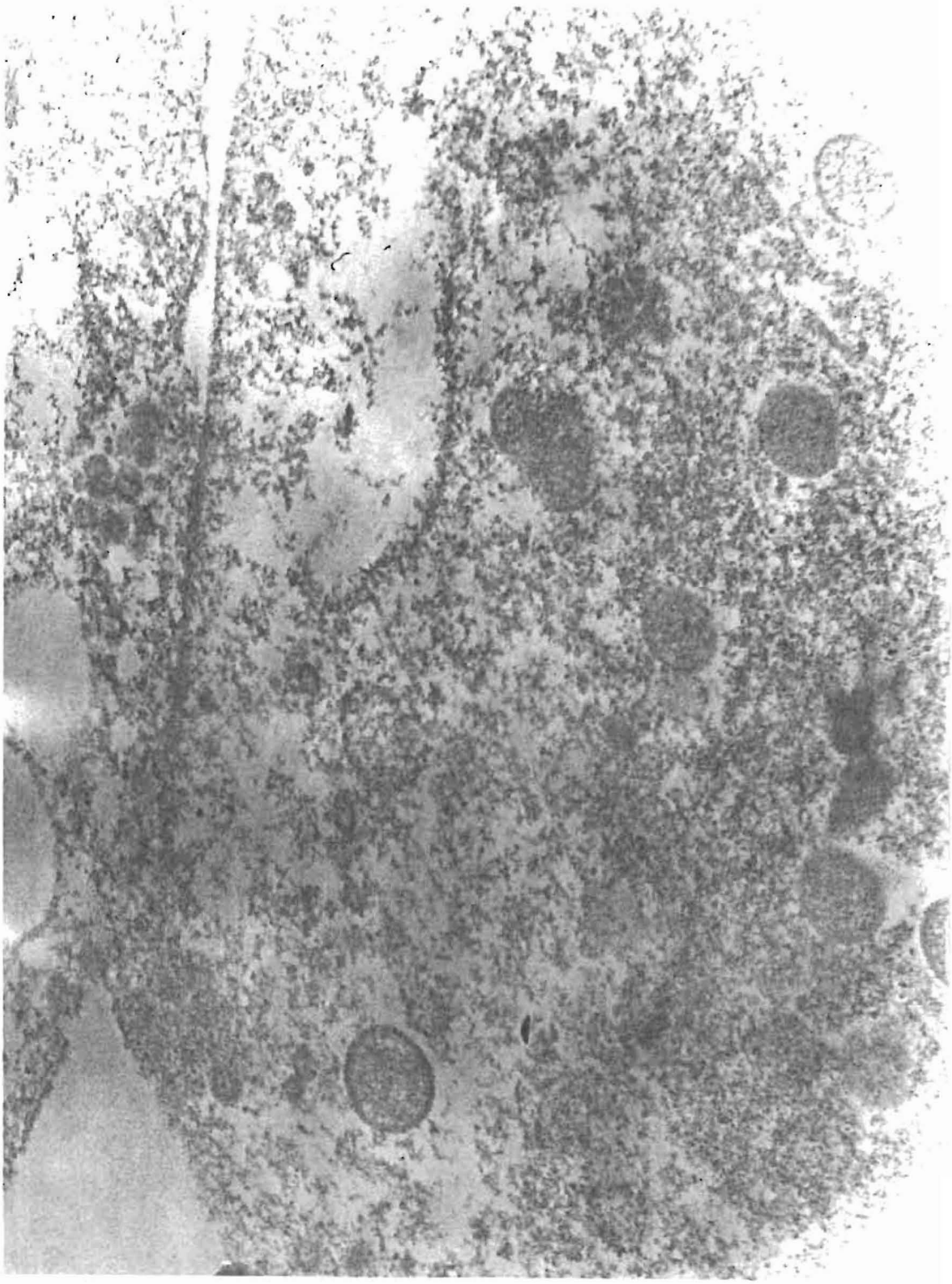


Fig. 16. Electron micrograph of infected mouse peritoneal macrophage after 12 hours. (X 54,000) Uranyl acetate/lead citrate stain.

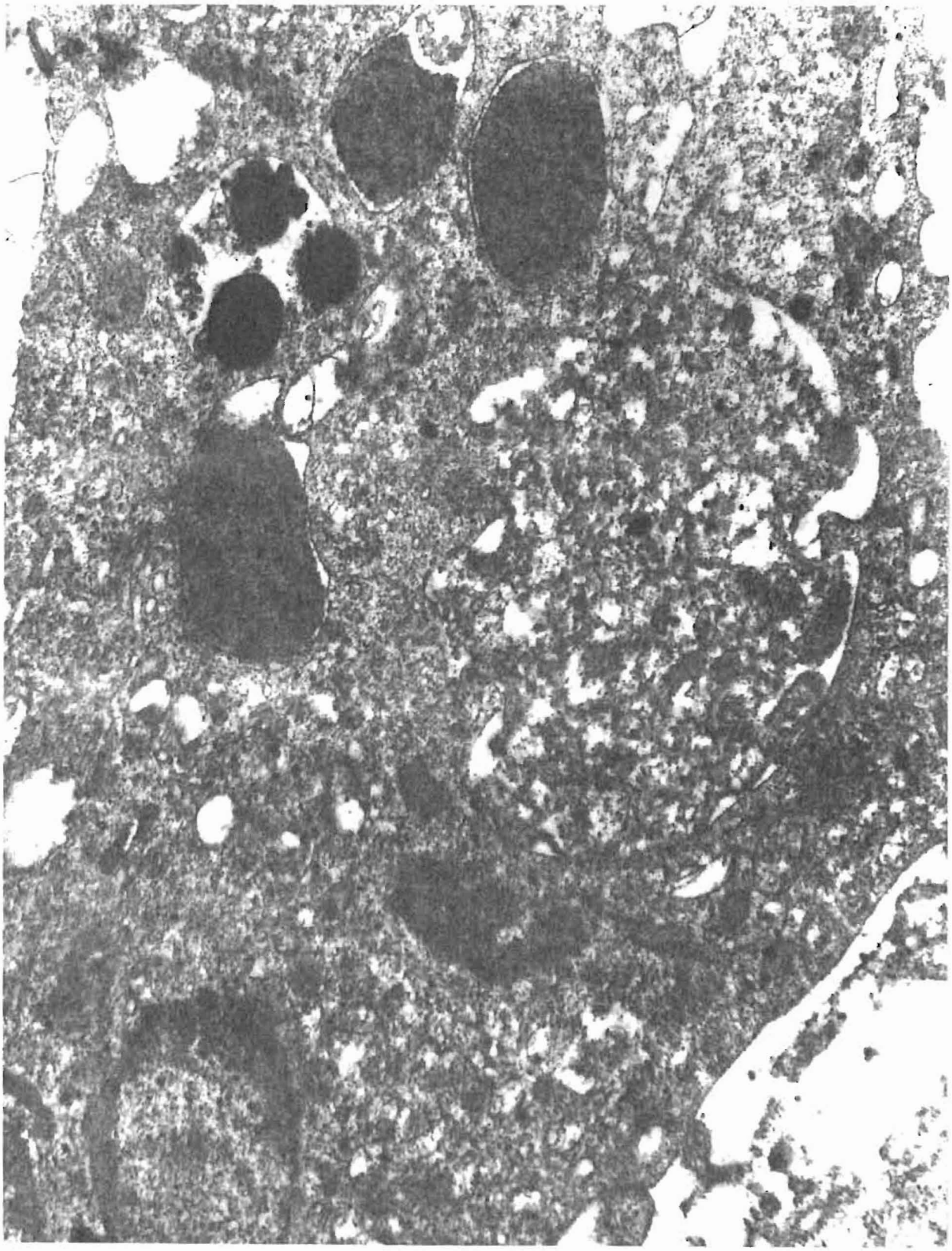


Fig. 17. Infected rabbit peritoneal macrophage after 12 h incubation. Immature forms of vaccinia (v) are evident in viroplasm. (X 56,000) Uranyl acetate/lead citrate stain.

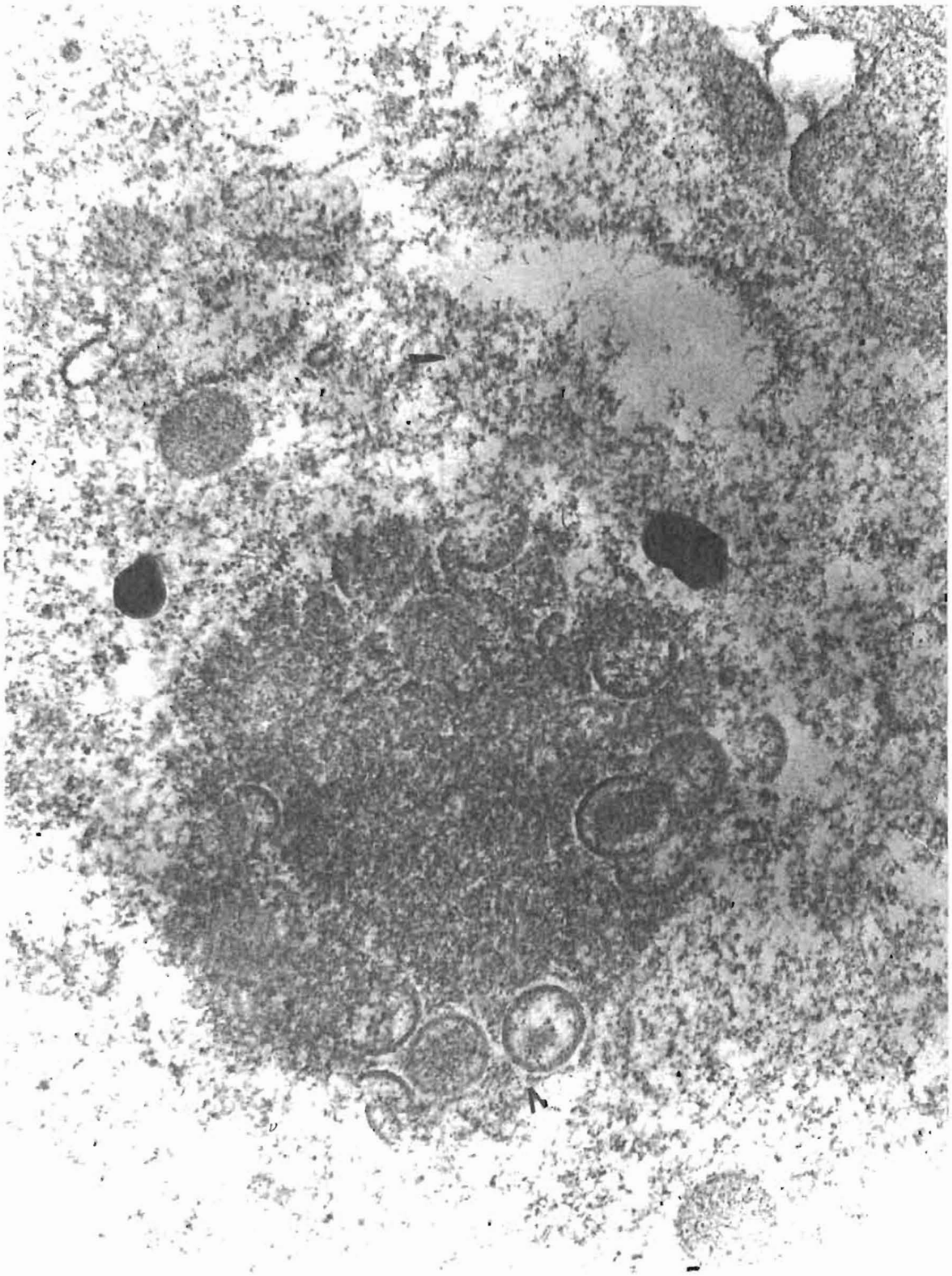


Fig. 18. Electron micrograph of infected mouse macrophage after 24 h incubation. No viral replication is observed. (X 54,000) Uranyl acetate/lead citrate stain.

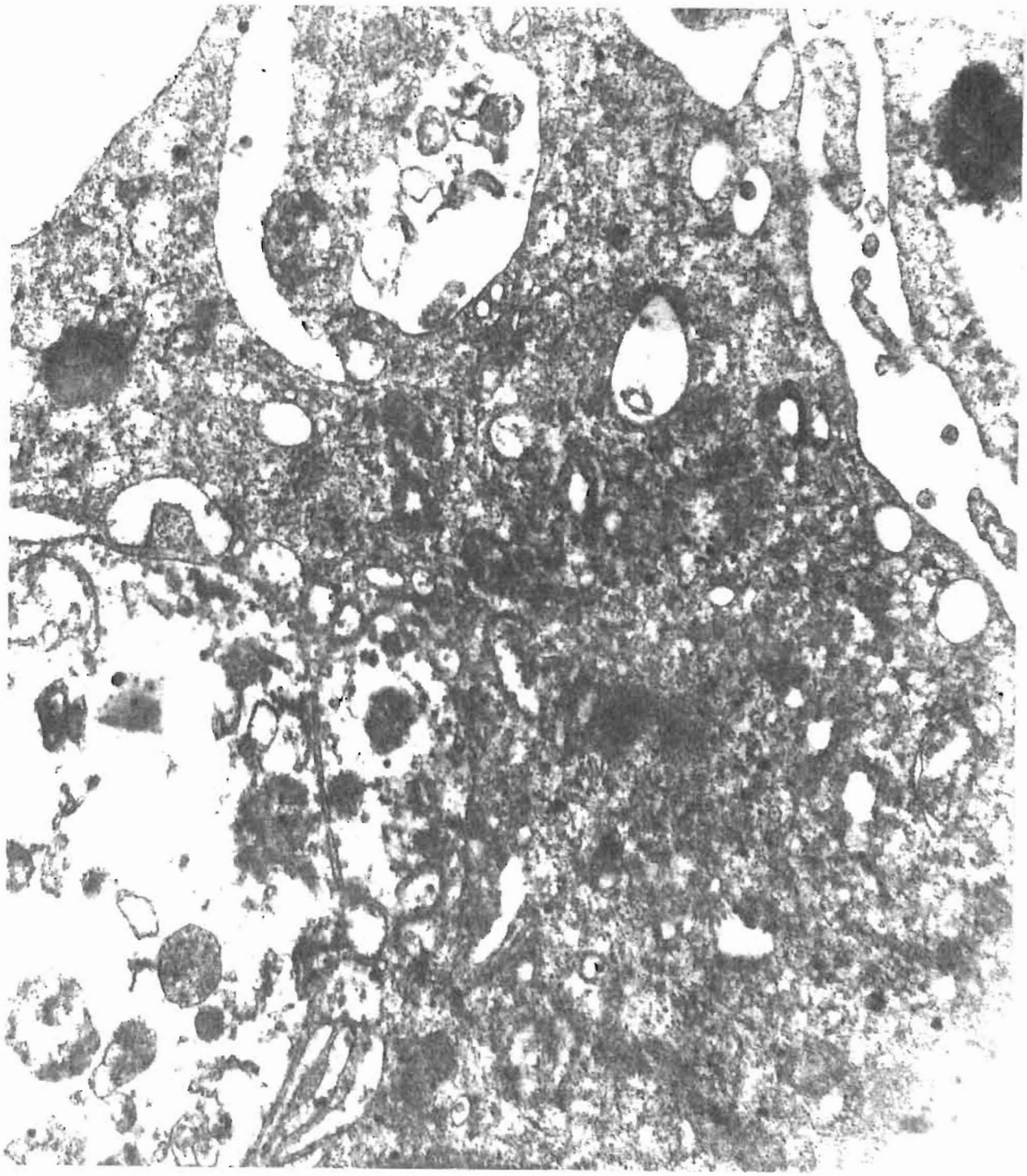


Fig. 19. Electron micrograph of infected rabbit macrophage after 24 h incubation. Maturing particles of vaccinia, some showing more condensed DNA (V), can be seen in the cytoplasm. (X 54,000) Uranyl acetate/lead citrate.

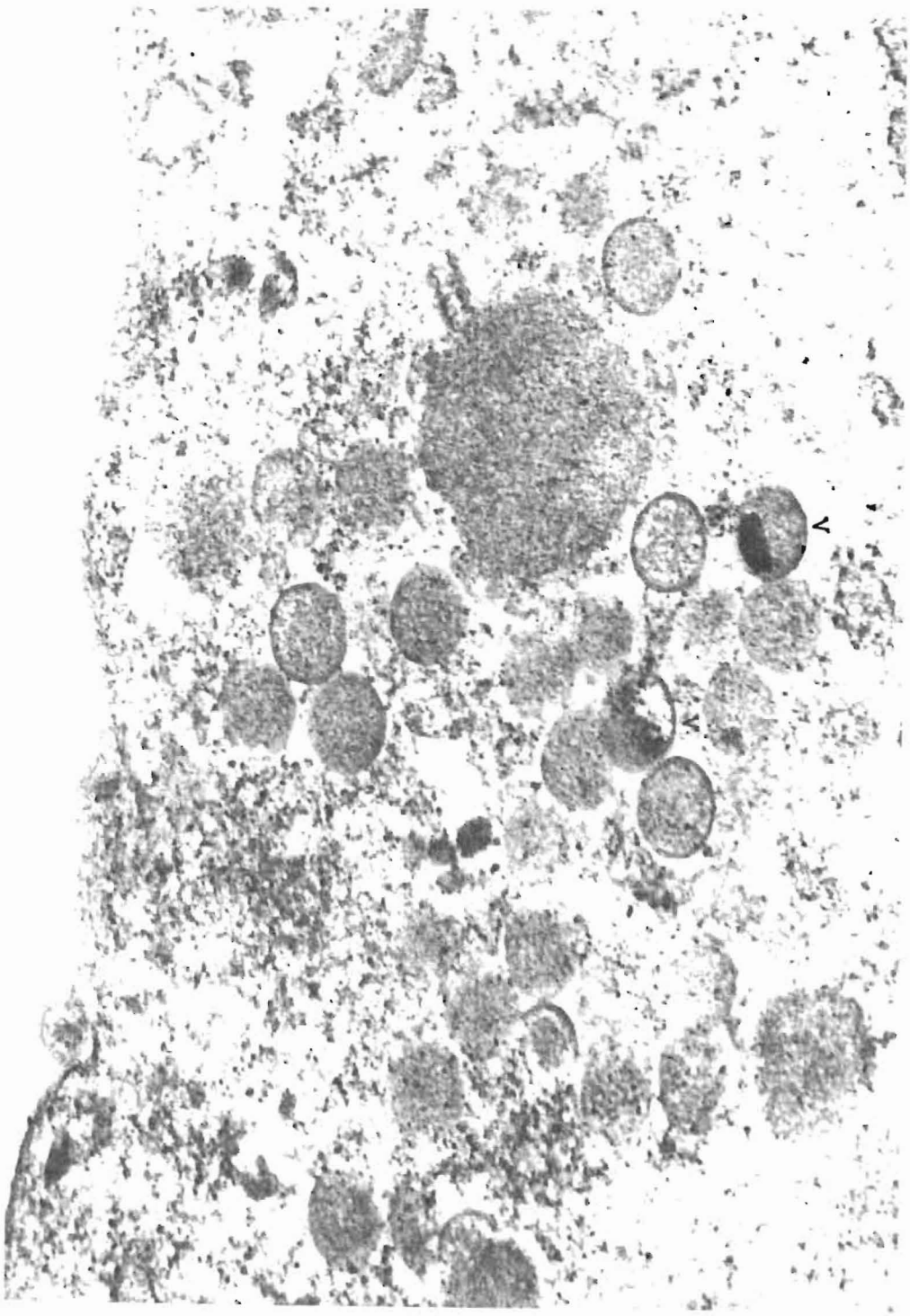


Fig. 20. Infected mouse peritoneal macrophage after 24 h incubation showing no viral replication. (X 54,000) Uranyl acetate/lead citrate stain.

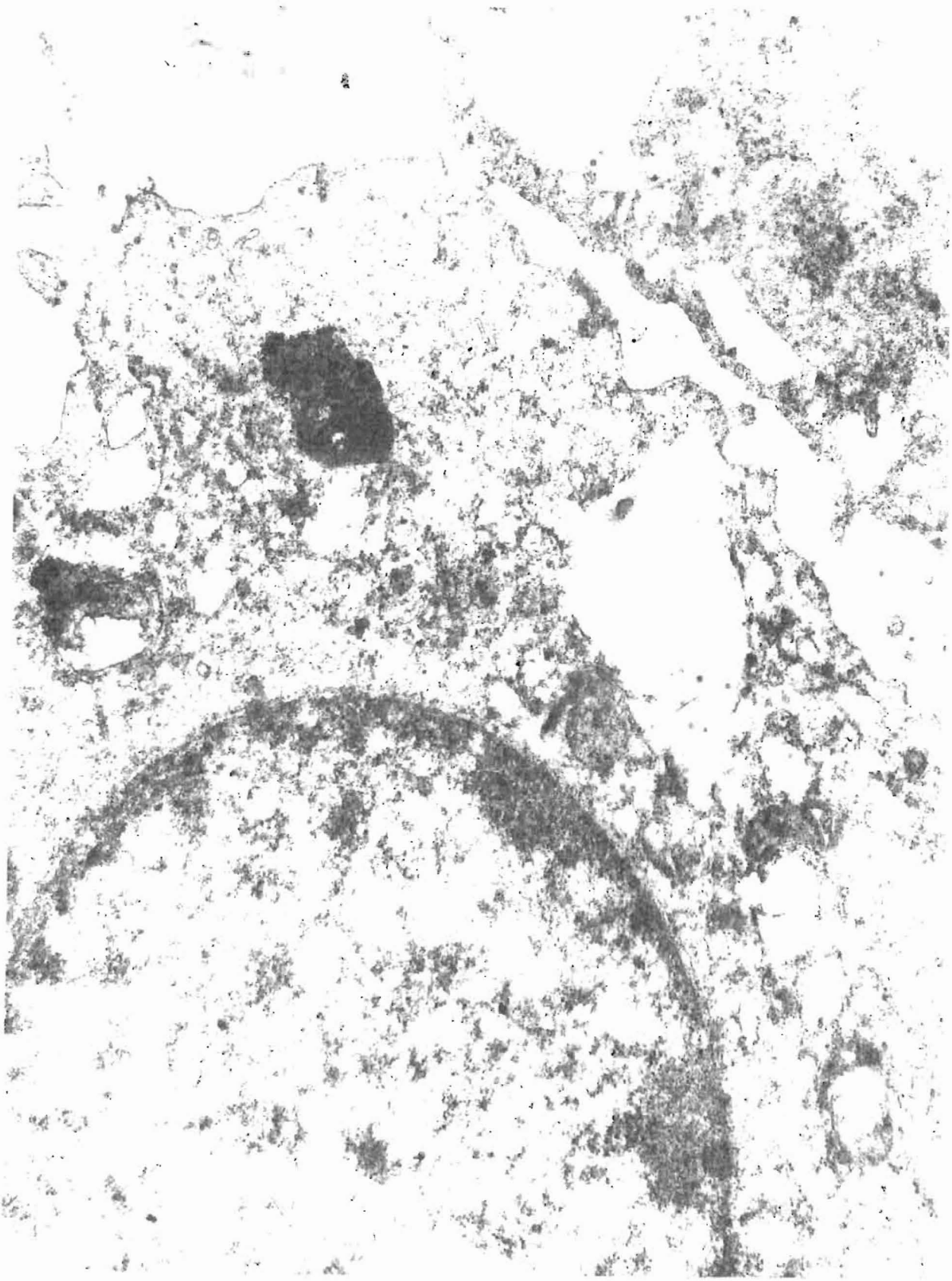


Table 2. Number of areas of viral reproduction per 100 sections of mouse and rabbit macrophages.

EVIDENCE OF ABORTIVE INFECTION		
Time	Number of viral factories	
	Rabbit	Mouse
12 hrs	23	0
24 hrs	35	0

Table 2. Number of areas showing viral reproduction (viral factory) per 100 sections of macrophages.

Biochemical tests

1. Second stage of uncoating

The second stage of uncoating was assayed by monitoring the amount of ^{3}H-thymidine labeled viral DNA which became accessible to DNase. At 30 min post infection, the acid soluble supernatant from both systems contained little radioactivity, indicating no sensitization of DNA to DNase, therefore no second stage of uncoating (Tables 3, 4). At 4 and 8 h post infection, the viral DNA became DNase sensitive, as evidenced by the higher radioactivity count in the supernatant than in the precipitate, thus confirming the occurrence of the second stage of uncoating in both mouse and rabbit macrophages.

In contrast, little radioactive material was acid soluble when the samples were incubated without DNase. This indicated that the high radioactivity count in the acid soluble supernatant was not due to the destruction of vaccinia virus by the cells.

2. Thymidine Kinase activity

Thymidine kinase activity was measured by determining the amount of ^{3}H-thymidine which became phosphorylated and, therefore bound to ion exchange paper. The results indicated that there was little phosphorylation of thymidine in uninfected rabbit macrophages (Fig. 21). Infected mouse macrophages, like the uninfected controls, showed little thymidine phosphorylating activity. In contrast, infected rabbit macrophages showed a linear increase in thymidine kinase activity up to 12 h post infection, followed by a slight decrease at 24 h post infection.

Table 3. Accessibility of radiolabeled vaccinia virus DNA to DNase at different times following infection of rabbit macrophages.

Table 4. Accessibi;ity of radiolabeled vaccinia virus Dna to DNase at different times following infection of mouse macrophages.

Table 3. Assay of second stage of uncoating in rabbit macrophages at 30 min, 4 h, and 8 h postinfection.

	Rabbit Macrophages			
	Lysate + TCA		Lysate + TCA + DNase	
Time	Supernatant	Pellet	Supernatant	Pellet
30 min	31.8 %	68.2 %	36.3 %	64.7 %
4 hrs	35.1 %	64.9 %	65.6 %	34.4 %
8 hrs	37.7 %	62.3 %	74.6 %	25.4 %

Table 4. Assay of second stage of uncoating in mouse macrophages at 30 min, 4 h, and 8 h postinfection.

	Mouse Macrophages			
	Lysate + TCA		Lysate + TCA + DNase	
Time	Supernatant	Pellet	Supernatant	Pellet
30 min	23.6 %	76.4 %	36.1 %	63.9 %
4 hrs	23.4 %	76.6 %	70.0 %	30.0 %
8 hrs	23.5 %	76.5 %	60.3 %	39.7 %

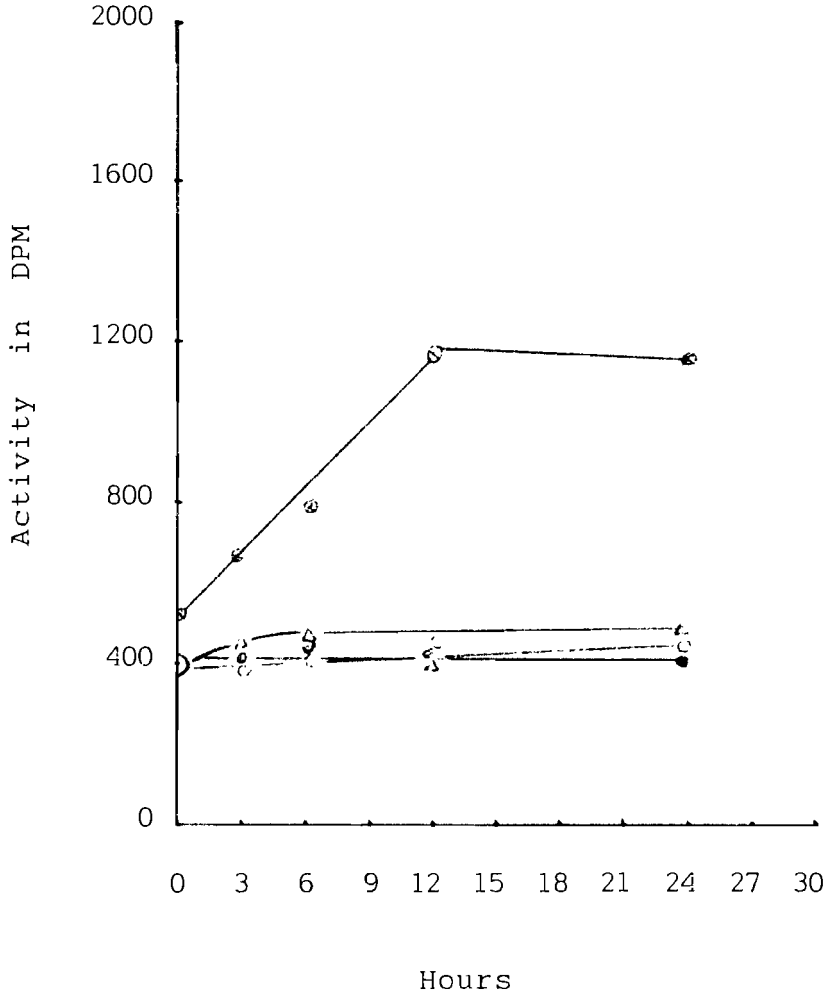
Fig. 21. Thymidine kinase activity in mouse and rabbit macrophages infected up to 24 h with IHD vaccinia virus.

O = infected mouse

⊗ = infected rabbit

● = uninfected mouse

▲ = uninfected rabbit


2000
1600
1200
800
400
0
Activity in DPM
0 3 6 9 12 15 18 21 24 27 30
Hours

DISCUSSION

Macrophages play an important role in host resistance or susceptibility to viral infections. They also display an extensive array of immunological functions. For the most part, these functions are inducible rather than constitutive, and the processes leading to their expression may be referred to collectively as "macrophage activation" (Meltzer et al. 1979; Nancy et al. 1984; Friedman and Beller 1987). In an infectious process where the outcome depends on the race between multiplication of virus in the primarily infected cells on the one hand, and establishment of the immune response on the other, phagocytosis leading to virus destruction is of crucial importance.

Naturally occurring resistance of mouse peritoneal macrophages to vaccinia virus has been well documented (Nishmi and Bernkopf 1950; Nishmi and Niecikowski 1963; Schultz 1966; Milligan 1983; Clark 1984; Kashanchi 1986). In view of these findings, it is important to point out that vaccinia virus has no apparent effect upon adult mice unless given intravenously in very large doses (Zakoy-Roness et al. 1962). Infection of mouse macrophages with herpes virus results in a abortive infection. There is an accumulation of virus antigens in the cell but no complete infectious virus is produced (Stevens and Cook 1971). In the case of vaccinia virus, it enters the cell cytoplasm but is unable to produce infectious progeny (Avila et al. 1972; Kashanchi 1986). The data presented in this thesis confirm and extend these findings.

Inoculation of peritoneal macrophages from nonimmune rabbits with vaccinia virus resulted in a characteristic vaccinia-induced cytopathogenic effect on the macrophage culture. In contrast, cultures of normal mouse macrophages were free of viral injury, confirming the ability of mouse macrophages to abort vaccinia virus infection. The observation that the amount of cell-associated virus at the end of the adsorption period is similar in both systems (Greer 1973; Schultz 1966; Milligan, 1983; Kashanchi 1986) argues against differences in capacity of the macrophages to adsorb virus as the operative factor in the observed resistance or susceptibility to vaccinia infection.

Electron microscopy revealed no difference in the efficiency of viral uptake between mouse and rabbit macrophages (Kashanchi 1986). Mouse macrophages were able to not only phagocytize the virus as efficiently as rabbit macrophages (Greer 1973), but they also processed it through the first stage of uncoating as indicated by the presence of viral cores in the cell cytoplasm. Therefore, inhibition of viral replication in mouse macrophage could not be due to the digestion of the virus by lysosomal enzymes as suggested by Schultz (1966), Clark (1984), and Milligan (1983), or failure of the mouse to ingest the virus.

Kashanchi (1986), using suramin as a host lysosomal stabilizer, showed that there was no increase in infectious viral titer in rabbit macrophages. Similar observations were made by Joklik (1966) and Dales (1963) using Hela cells. Hence, it was concluded that while lysosomal enzymes did not contribute to destruction of vaccinia virus in mouse macrophages, they were nevertheless needed for the first stage of uncoating. Future studies will determine the means by which viral

cores gain access to the macrophage cytoplasm.

The observed increase in the number of cytoplasmic cores from 0 to 2 h post infection suggested that the first stage of uncoating took place in both cell types as the cores accumulated in the cell cytoplasm (Table 1). The decrease in the number of cores from 2 to 6 h post infection suggested the release of naked viral genome and the second stage of uncoating. This was confirmed by assaying for the amount of viral DNA which became accessible to DNase hydrolysis. In the absence of DNase, however, supernatant samples showed consistently low radioactivity. Hence it was concluded that the observed DNase sensitivity of the viral genome could not be due to destruction of the virus by mouse macrophages. The occurrence of the second stage of uncoating in mouse macrophages indicated that some transcription and translation, specifically synthesis of the uncoating enzyme, took place. However, mouse macrophages prevented the synthesis of other proteins as evidenced by the fact that subsequent events in the virus replication cycle were not observed.

Quantitation of virus factories revealed that at 12 hours post infection approximately one out of four macrophages in the nonimmune rabbit cell population showed immature forms of vaccinia virus at 24 h post infection and nearly one out of three rabbit macrophages observed showed maturing forms of vaccinia virus (Table 2). In contrast, no evidence of viral replication was ever observed in mouse macrophages. These observations provided visual evidence for viral replication in rabbit macrophages (Figs. 17, 19) and lack of viral replication in mouse macrophages (Figs. 18, 20).

Because the second stage of uncoating was observed in both cell types, thymidine phosphorylation was assayed to determine if more early proteins were synthesized. Thymidine kinase is one of the earliest enzymes observed in vaccinia virus infection (Kit et al. 1962; Sheek 1961; McAuslin 1963; Esteban and Metz 1972). Therefore, it was used as a marker of early gene function.

Uninfected mouse and rabbit macrophages, as well as the non-cellular enzyme control, consistently showed low thymidine kinase activity (Fig. 21). In experiments carried out through 24 h postinfection, rabbit macrophages showed a linear increase in thymidine phosphorylating activity up to 12 h, followed by a slight decrease. In contrast, no significant thymidine kinase activity was observed in mouse macrophages.

This evidence suggests that normal mouse peritoneal macrophages express innate resistance against vaccinia virus by suppressing the synthesis of early proteins such as thymidine kinase (used only as a marker) which leads to the suppression of viral DNA synthesis. At the present time, we offer no explanation on the precise mechanism that may be operable. My work shows that interferon could not be the operating factor, as proposed by Kashanchi (1986).

Bodo et al. (1979) showed that interferon selectively inhibits vaccinia-specific translation. Interferon-treated cells synthesize early viral ribonucleic acid but no early viral DNA (Lewine et al. 1967). Interferon also inhibits the second stage of uncoating of vaccinia virus (Magee et al. 1968), and yet I found visual evidence of the disappearance of viral cores as well as biochemical results indicating the occurrence of the second stage of uncoating.

Furthermore, several researchers (Tompkins et al. 1970; Subrahmyan and Mims 1970) failed to show enhanced interferon-like activity in macrophages from vaccinia virus-resistant mice in comparison to the susceptible mice.

The possibility exists that mouse macrophages can synthesize thymidine kinase, but in an inactive form or perhaps it requires substances not present in the subtrate mixture that we used. This problem can be solved by treating cell free lysates of vaccinia-infected mouse and rabbit macrophages at different time intervals with immunoglobulins from antivaccinia virus thymidine kinase and looking for a precipitation reaction as a sign of thymidine kinase production.

Future research will determine whether thymidine kinase, or any other early protein for that matter, is inhibited at the transcription or translation level. This information will allow us to gain more insight into the intracellular mechanisms of innate resistance of mouse macrophages to vaccinia virus.

SUMMARY

1. Electron micrograph studies revealed an increase in the number of cytoplasmic viral cores in both mouse and rabbit macrophages up to 2 h post infection, thus substantiating the occurrence of the first stage of uncoating in both cell types.
2. The observed decrease in the number of cores from 2 to 6 h post infection suggested the release of naked viral genome and the occurrence of the second stage of uncoating.
3. Biochemical tests revealed that viral DNA was converted to DNase-sensitive form in both the susceptible rabbit macrophages and the resistant mouse macrophages, thus confirming the occurrence of the second stage of uncoating.
4. Electron micrographs of late infection revealed immature forms of vaccinia virus in rabbit macrophages. In contrast, no evidence of viral replication was observed in mouse macrophages, indicating that viral replication was blocked early in this virus-macrophage interaction.
5. Thymidine kinase assay revealed no significant amount of thymidine kinase activity in mouse macrophages in comparison to rabbit macrophages. These data suggest inhibition of early virus-induced enzymes by mouse macrophages as a means of expressing innate resistance against vaccinia virus.

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