AN ABSTRACT OF THE THESIS OF

Vickie E. Clark for the Master of Science Degree

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Title: Mechanisms of Genetically Endowed Macrophage Resistance

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An electron microscope study of the interaction of vaccinia virus with various peritoneal macrophage systems was conducted. Normal rabbit peritoneal macrophages allowed first stage uncoating to proceed; viral cores were present within the cellular cytoplasm. Normal mouse peritoneal macrophages did not allow viral core formation and appeared to degrade the virus within phagocytic vacuoles. Thioglycollate-elicited mouse peritoneal macrophages allowed cytoplasmic core formation. Virus that was not uncoated appeared to be undergoing degradation within the phagocytic vacuole within six hours after infection.

Results of this study suggest that the state of cellular activation may control the fate of vaccinia. Furthermore, the level of lysosomal enzymes, especially acid phosphatase, seem to be important in determining whether the virus undergoes replication or degradation.

MECHANISMS OF GENETICALLY ENDOWED MACROPHAGE RESISTANCE

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To Ann Scheve a special thanks is due for helping me in countless ways with my research. Also to Roger Ferguson who kept me supplied with animals I wish to express my appreciation.

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INTRODUCTION

When an animal is invaded by a foreign organism, the host's immune system mounts a response. The first cells of the immune system to encounter the foreign material are the phagocytic cells. Metchnikoff (1905) first described the phagocytic cells as specialized leukocytes within the body which performed acts of phagocytosis of foreign substances. The macrophages, found in tissues and body cavities, were so named because they were "big eaters," that is, a single cell could engulf large cells such as erythrocytes. The microphages or "small eaters" were also described as phagocytic cells but they ingested smaller organisms such as bacteria. He also recognized the close similarities between the macrophages in the spleen, liver, lymph nodes and bone marrow with those located outside these organs in the connective tissue.

This concept of grouping similar phagocytic cells together was carried further. Aschoff (Langevoort, et al, 1970) grouped several kinds of cells together into the Reticulo-Endothelial System. This classification notes the differences in morphology and rate of phagocytosis between the various types of phagocytic cells. In light of the current knowledge of morphology, kinetics, and function, all highly phagocytic mononuclear cells and their precursors can now be placed in a single system. The proposed name is the mononuclear phagocyte system (MPS) (Langevoort, et al, 1970; van Furth, 1975).

The morphology of the mononuclear phagocyte is dependent on the organ or tissue the cell is harvested from. Macrophages may be difficult to identify on the basis of staining alone. These cells have much more extensive cytoplasmic ruffling than is seen with other cell types.

The primary criteria that distinguish the mononuclear phagocytes from other cells are avid phagocytosis and pinocytosis, especially the former, and firm attachment to glass surfaces (van Furth, 1975).

The phagocytic cells in the MPS are all derived from a stem cell which is present in the bone marrow. Here the stem cell undergoes differentiation and maturation. The stem cell leaves the bone marrow and enters the blood as a monocyte. While in the circulation, the monocyte undergoes further differentiation, then leaves the blood and enters tissues and body cavities where it finally differentiates into a mature macrophage (Nichols and Bainton, 1975). Tissue culture studies suggest that the majority of blood monocytes possess the capacity for transformation into macrophages (Vernon-Roberts, 1972). The monocytes leave the circulation in a random manner. About 56 % become Kupffer cells in the liver, about 15 % become pulmonary macrophages, and about 8 % become peritoneal macrophages (van Furth, et al, 1982). Once the macrophage is fully mature it may persist virtually unchanged for a very long time, possibly years.

"Resident macrophages" refers to the population which is obtained from a particular anatomic site without experimental elicitation and in the absence of any known endogenous inflammatory agent. "Elicited macrophages" refers to the accumulation of macrophages at a particular anatomic site, but does not necessarily imply a functional alteration in cellular activity. Occasionally, variations may be present in the amount of cellular activity of elicited macrophages. "Activated macrophages" refer to those macrophages that are activated by immunologic mechanisms in vivo for enhanced anti-microbial activity (Morahan, 1980; van Furth, et al, 1982).

Elicited macrophages also have been reported to have increased metabolic activities. Casein-elicited guinea pig peritoneal macrophages have been shown to have higher respiratory rates than normal peritoneal macrophages (Karnovsky, et al, 1970). Furthermore, elicited macrophages show an increase in respiration and oxidative activity toward glucose during phagocytosis. Normal mouse peritoneal macrophages washed from the peritoneal cavity without the use of an eliciting agent show no increased respiratory activity during phagocytosis; elicited mouse macrophages show remarkable respiratory activity (Karnovsky, et al, 1970). Mineral oil elicited rabbit peritoneal macrophages contain a level of lysosomal hydrolases that is slightly higher than normal un-elicited macrophages (Cohn, 1970).

Macrophages are in a position to control the susceptibility of animals to viral infections since they monitor the main body compartments and may also control the entry of virus to target organs such as the liver. Mims (1964) has summarized evidence that viruses introduced into the lungs or other anatomical sites are taken up by macrophages in local tissue and lymph nodes. As a general rule, viruses cannot multiply sufficiently in macrophages to spread to other susceptible cells. However, if the macrophage barrier is bypassed and the susceptible cells are exposed to the virus, a lethal infection with widespread multiplication of virus is the result. He also showed evidence that correlated the resistance of macrophages with a number of viruses and resistance of the whole animal to that same agent.

This correlation of resistance is supported by several investigations. Bang and Warwick (1960), working with mouse hepatitis virus (MHV) showed that resistance was dependent on the genetic strain of the

mouse. Resistance was related to the ability of the resistant mouse macrophages to maintain the virus in a non-replicative state within the cell. Susceptibility of mice to MHV was not dependent on the age of the animal or age of the culture (Kantoch, et al, 1963).

Host resistance or susceptibility to many viruses has been shown to be genetically determined. Bang and Warwick (1960) showed that mice which displayed susceptibility to MHV also had macrophages that were susceptible to MHV. They concluded that susceptibility was the result of a single dominant gene. This conclusion was confirmed by Kantoch, et al (1963) also working on MHV. Lindenmann, et al (1978) working with myxovirus, found that resistance to the virus followed patterns of genetic inheritance that would be expected with a single autosomal dominate gene. Mogensen (1979) presented evidence that inherited resistance to herpes simplex virus type 2 (HSV-2) is by a single X-linked dominant gene. However, Lopez and Dudas (1979), working with herpes simplex virus type 1 (HSV-1), were unable to establish a correlation between genetic resistance of the animal to the virus and the resistance of the macrophage to the virus. Rather, they showed that the state of activation of the macrophage played a major role in macrophage resistance to HSV-1. Unstimulated macrophages from resistant mice were able to suppress HSV-1 replication, whereas proteose-peptone or thioglycollate-elicited peritoneal macrophages from resistant mice allowed proliferation of the virus. This phenomenon may be important in other virus-macrophage systems as well. Mouse peritoneal macrophages, which are normally resistant to vaccinia virus replication, allowed at least first stage uncoating of the virus when peritoneal macrophages were elicited by the injection of thioglycollate (Silverstein, 1975).

Milligan (1983) demonstrated no vaccinia virus replication in normal mouse macrophages. This may indicate that the level of activation of the macrophage plays a crucial role in the replication of vaccinia virus, at least in the mouse macrophage system.

Schell (1960a), working with the mousepox and ectromelia virus showed that resistance was dependent on the genetic strain of mice. He demonstrated that resistant adult mice produced more neutralizing antibody than susceptible mice. Furthermore, he found that the production of antibody was dependent on the route of inoculation with antibody production highest following intravenous injection (Schell, 1960b). Lindenmann, et al (1978) working with myxovirus also demonstrated that the expression of genetically endowed macrophage resistance was correlated with the age of the animal. Newborn mice that were genetically resistant animals were susceptible to the virus while adults were resistant. Lopez (1975) showed that newborn mice, which were genetically resistant to herpes simplex virus, allowed viral replication when inoculated with the virus. A significant number of these gentically resistant but immature mice died as a result of the viral infection again emphasizing the importance of age.

However, resistance to viruses is not always dependent on the age of the host. Bang and Warwick (1960) showed that newborn mice were resistant to MHV. Work in this laboratory (Milligan, 1983) showed that mouse macrophages were resistant to vaccinia virus at least by the fourth week of life.

Frequently, newborn animals display low resistance to viral infections; however, there are many exceptions. Weak resistance is probably due to immaturity of the immune system, especially the immaturity of

the macrophage system. In humans, an example of macrophage immaturity is seen with rubella virus infection of the fetus. An infant infected in utero shows a variety of characteristic symptoms and pathology known as the rubella syndrome. Among these are deafness, cataracts, cardiac abnormalities, anemia and low birth weight (Ginsberg, 1980). Although these infants develop specific humoral antibody against rubella virus, they may continue to excrete virus in nasopharyngeal secretions for as long as two years (Glasgow, 1970; Ginsberg, 1980). As the child's macrophages mature, the virus is no longer shed and the child may develop protective immunity to rubella virus.

Greer, et al (1974) showed with the electron microscope that peritoneal macrophages from vaccinia immunized rabbits were as active in phagocytosing vaccinia virus as were macrophages from the non-immune rabbit. They showed the immune macrophage allowed viral first stage uncoating followed by core formation in the cytoplasm. Buchmeier, et al (1979) in agreement with these results, also found core formation in vaccinia immune rabbit macrophages. They showed that replication of vaccinia virus was halted at a late point in the replicative cycle during viroplasmic condensation. Ward (1980), working with thioglycollate-elicited mouse peritoneal macrophages, and Milligan (1983), working with normal mouse peritoneal macrophages, have both demonstrated the absence of active DNA formation in vaccinia virus infected macrophages; therefore, it appears that the mouse macrophage system is capable of suppressing vaccinia virus replication in a most efficient manner.

The purpose of this research was to examine, by use of the electron

microscope, normal rabbit macrophages and normal and thioglycollate-elicited mouse peritoneal macrophages which have been infected with vaccinia virus, in an attempt to determine the fate of vaccinia virus. Normal rabbit macrophages allow replication of vaccinia virus while normal mouse macrophages suppress vaccinia replication. The goal was to determine the fate of vaccinia virus in thioglycollate-elicited mouse peritoneal macrophages, to learn whether or not viral replication was allowed thus providing information in regard to the non-immune mechanisms of macrophage resistance to virus infection.

MATERIALS AND METHODS

Experimental Animals

Mice were obtained from the Emporia State University Division of Biological Sciences animal facility. Rabbits were obtained from local breeders.

Cell Culture Media

Mouse and rabbit peritoneal macrophages were harvested in Eagle's minimal essential media (MEM) containing two units of heparin per ml and 2 % newborn calf serum (Kansas City Biological). Mouse embryo fibroblasts were harvested in MEM. All cell types were cultured in MEM and were supplemented with newborn calf serum at a final concentration of 10 %. Combiotic (Penicillin G, 200 units/ml; Streptomycin, 100 units/ml) was added to all cell harvest and culture media. Sterile 1.5 % or 7.5 % sodium bicarbonate was used to adjust the pH of the cultures when necessary.

Mouse Embryo Fibroblasts

Pregnant females were sacrificed by cervical dislocation on the tenth to sixteenth day of gestation. The fur was wetted with disinfectant and the abdomen was aseptically opened. Both horns of the uterus were removed and placed in a sterile petri dish containing MEM and combiotic. The embryos were removed from the uterus and were decapitated and eviscerated. Blood and loose tissue were removed by three successive washes in sterile MEM. The embryos were placed in a trypsinizing flask containing a sterile pronase solution (0.125 g pronase in 50 ml of MEM; Sigma Chemical Company, St. Louis, MO). The cells were dispersed for two hours at room temperature by stirring on a magnetic stirrer. Five ml of sterile serum was added to the flask

and the cells were stirred for an additional five minutes. The cells were poured through a sterile cheesecloth filter to remove debris and cell clumps. The resulting cell suspension was washed three times with MEM plus combiotic, and counted with an improved Neubauer hemocytometer. The suspension was adjusted to contain 2 X 10^6 cells per ml. The cells were then planted in Roux flasks for virus propagation or tissue culture flasks for virus titration.

Mouse Peritoneal Macrophages

To obtain elicited macrophages, adult mice were injected intraperitoneally with two ml of 3 % thioglycollate four days before harvesting. Nonelicited macrophages were obtained from normal adult mice. The mice were sacrificed and the cells were harvested using standard aseptic technique. Harvests from five to ten mice were pooled.

The cell suspension was washed three times in MEM plus combiotic, counted and adjusted to 4 X 10^6 cells per ml. Cells were planted in four ml quantities in tissue culture flasks for electron microscope work or one ml quantities in flying coverslip tubes for light microscopic observation. The cells were allowed to incubate for one to two hours at 37°C. After gentle agitation the supernatant containing nonadherent cells was removed with a sterile Pasteur pipette and replaced with MEM plus combiotic and 10 % newborn calf serum.

Rabbit Peritoneal Macrophages

Five days prior to harvest, rabbits were injected intraperitoneally with 50 ml of sterile mineral oil. For harvest, the rabbits were sacrificed by air embolism. The fur was wetted with disinfectant and the abdominal skin was laid back. The abdominal muscle wall was swabbed with disinfectant. One hundred ml of sterile MEM plus combiotic

was injected into the peritoneal cavity. The abdomen was gently massaged to suspend the resident peritoneal macrophages. The abdominal cavity was aseptically opened. Fluid was removed with a 10 ml pipette and placed in a sterile separatory funnel. After completion of the harvest, the oil and aqueous phases of the harvest were allowed to separate at 4°C for ten minutes. The aqueous phase containing cells was pelleted by centrifugation, washed, counted, and planted as described.

Virus Propagation

The IHD strain of vaccinia virus used in this study was originally obtained from the American Type Culture Collection. It was propagated in mouse embryo fibroblast monolayers.

Roux flasks containing mouse embryo fibroblast monolayers were washed twice with sterile Dulbecco's phosphate buffered saline (DPBS). The monolayer was infected by adding 15 ml of MEM containing 10^4-10^5 plaque forming units (PFU) of IHD vaccinia per ml. The virus was allowed to absorb to the monolayer for a two hour period. During this period the flasks were gently agitated every 15 minutes to facilitate absorption. The infecting medium was removed and the monolayer was again washed twice with DPBS. Fresh MEM containing combiotic and 10 % newborn calf serum were added, and the cultures were incubated until complete cell destruction was observed. Monolayer destruction was normally complete after 48 hours.

The virus was harvested by subjecting the infected culture to three cycles of freeze-thaw to release intracellular virus. Cell debris was removed by centrifugation. The resulting stock virus was frozen at -70°C.

Assay of Virus

Viral titer expressed as plaque forming units (PFU) per ml was obtained by assaying the stock virus on mouse embryo fibroblast monolayers. Mouse embryo cells were added to tissue culture flasks. They were washed twice with DPBS just prior to formation of a complete monolayer. Appropriate dilutions of IHD in MEM plus combiotic and 2 % serum were added to 1.0 ml amounts. The virus was allowed to absorb for a two hour period with gentle agitation every fifteen minutes. After the two absorption period, the MEM containing virus was removed and the cultures were again washed twice with DPBS, followed by the addition of MEM plus combiotic and 10 % calf serum. The flasks were incubated at 37°C until plaque formation was evident, usually 40-48 hours. Plaque counts were done in the standard manner.

Vaccinia Infection of Peritoneal Macrophages

Peritoneal macrophages were harvested and cultured as described. Two hours after planting, the cell cultures were washed with MEM plus combiotic to remove cell debris and unattached cells. Infection was initiated by addition of 1.0 ml MEM, 2 % serum and IHD vaccinia to flying coverslip tubes, and 2.0 ml to tissue culture flasks. The infecting MEM contained eight PFU per cell which was approximately 1.3 X 10^8 PFU/ml. A two hour absorption period followed during which time the cultures were gently agitated every fifteen minutes to facilitate viral attachment. Two, four, and six hours after initiation of infection, coverslips were removed and stained with May Grünwald-Giemsa. Tissue culture flasks containing infected macrophage monolayers were fixed and processed for electron microscope observation.

Concentration of Vaccinia Virus

Infection of macrophages for electron microscopic observation required the vaccinia virus to have a high titer. To concentrate the virus, it was centrifuged, using a fixed angle head, for 90 minutes at 4°C at 20,000 X g. The concentrated virus was then resuspended in the proper volume of sterile medium to give the desired titer. The virus was dispersed by sonification in a Raytheon Sonic Oscillator (Raytheon Company; Waltham, Mass.) at 0.4 amperes for five cycles of fifteen seconds each.

Dehydration and Embedding of Macrophages for Electron Microscopy

After absorption of virus, the macrophage monolayer was fixed in 3 % buffered glutaraldehyde at room temperature for one hour, followed by three washes for 15 minutes in phosphate buffer (pH 7.2) at room temperature. Post-fixation was in 1 % buffered osmium tetroxide (4°C, 90 minutes), followed by three rinses in distilled water. The macrophage monolayer was scraped from the tissue culture flask with a rubber policeman and the cells were pelleted by centrifugation. Liquid 2 % purified agar was added to the cell pellet. After the agar had hardened, it was cut into 1 mm^2 pieces with acetone cleaned razor blades.

Dehydration and infiltration of the macrophages were done at room temperature. The cells were first placed in 40 % ethyl alcohol for two washes of five minutes each. Successive washes were for ten minutes each in 60 %, 80 %, and 95 % ethyl alcohol. The final three washes were in 100 % ethyl alcohol for ten minutes each. Infiltration was initiated by three washes for ten minutes each in propylene oxide. After the third wash a working mixture of propylene oxide plus Epon-Araldite-DMP 30 (1:1 v/v) was added. After one hour an equal volume of

working mixture was added to the mixture already present for approximately four hours. Embedding was achieved by adding pure working mixture to plastic Beem capsules along with the tissue. The blocks were hardened for 16 hours in a 35°C oven and 24-36 hours in a 60°C oven.

Sectioning, Staining and Observing Macrophages

After the blocks were hard they were trimmed and sectioned on an LKB ultramicrotome (Stockholm, Sweden). The sections were picked up on 300 mesh copper grids. The sections were stained with 2 % aqueous uranyl acetate for twenty minutes. They were rinsed with freshly boiled distilled water then stained for three minutes in Reynold's lead citrate. The sections were once more rinsed in freshly boiled distilled water, dried, and stored for observation.

The sections were examined and photographed with a Hitachi HS-B Electron Microscope. Kodak contrast 3.25 X 4 inch projector slide plates were exposed and developed in D-19. The negatives were then enlarged on Ilford Multicontrast II paper.

RESULTS

Light Microscopic Observations

(1) Rabbit Peritoneal Macrophages

Within two hours after planting the peritoneal cellular exudate, the majority of cells attached to the glass surface of the flying coverslips were macrophages. Their staining characteristics were typical of peritoneal macrophages. An idented, kidney-shaped, dark staining nucleus was apparent, surrounded by a slightly granular appearing cytoplasm (Fig. 1). After attachment, the macrophages began extending pseudopods. After infection, with vaccinia virus no changes were seen in the macrophage morphology for two or four hours (Fig. 2 and 3). By six hours after infection the cells began assuming a slightly rounded appearance and clumped together (Fig. 4).

(2) Thioglycollate-elicited Mouse Peritoneal Macrophages

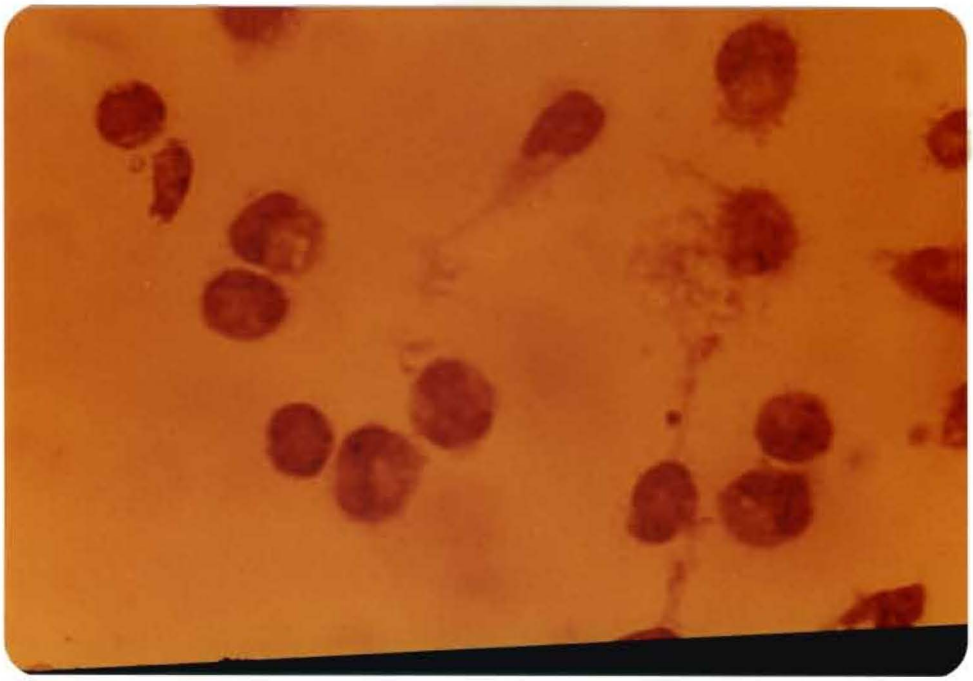
Harvest of the elicited peritoneal exudate yielded numerous lymphocytes as well as macrophages. Elicited macrophages showed typical peritoneal macrophage morphology, although they contained more numerous vacuoles than either the normal rabbit or mouse macrophages. Within twenty-four hours after planting, nearly all of the lymphocytes had been destroyed, leaving a population of macrophages (Fig. 5). At this time most macrophages had extended pseudopods. Two hours after infection with vaccinia virus, the macrophage cytoplasm appeared to be more rounded (Fig. 6). Four and six hours post-infection, the appearance of the macrophages was similar to that of two hours post-infection.

(3) Normal Mouse Peritoneal Macrophages

The normal mouse peritoneal macrophage attached to glass and spread in the characteristic manner, showing typical morphology (Fig. 7).

Fig. 1 Uninfected normal rabbit macrophages after two hours incubation (X 400) May Grünwald-Giemsa Stain.

Fig. 2. Normal rabbit macrophages infected with vaccinia virus for two hours. (X 400) May Grünwald-Giemsa Stain.



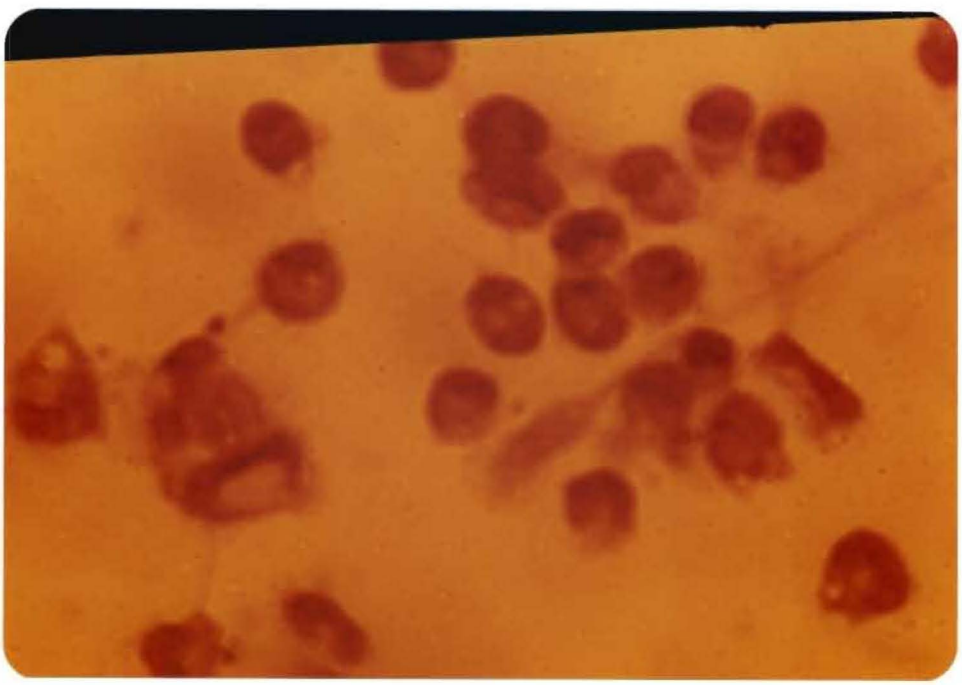
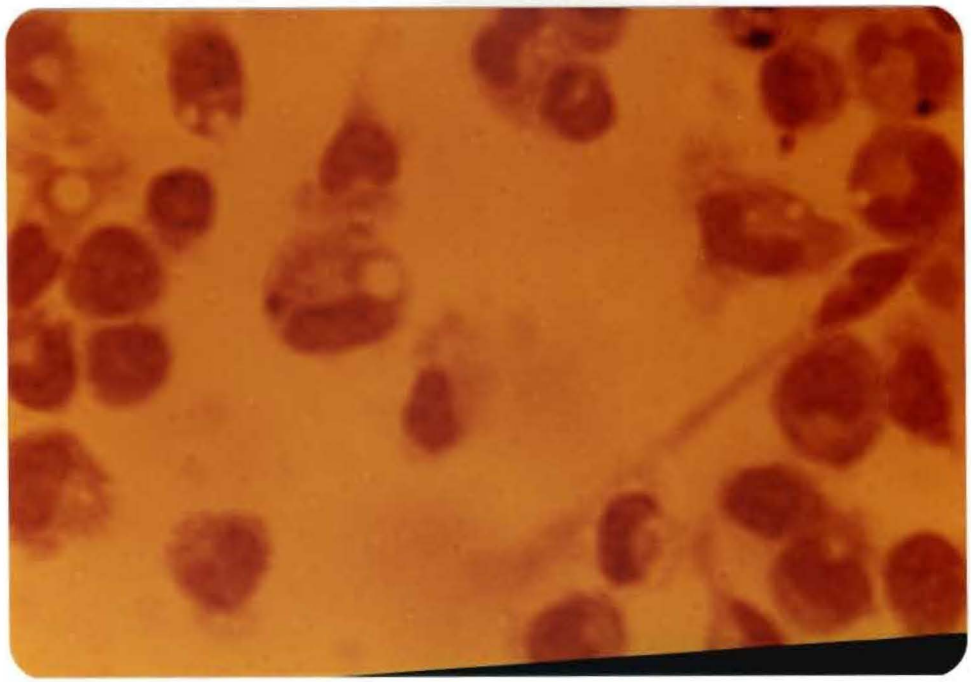


Fig. 3. Normal rabbit macrophages infected with vaccinia virus for four hours. (X 400) May Grünwald-Giemsa Stain.

Fig. 4. Normal rabbit macrophages infected with vaccinia virus for six hours. (X 400) Clumping of cells in typical viral CPE is exhibited. May Grünwald-Giemsa Stain.



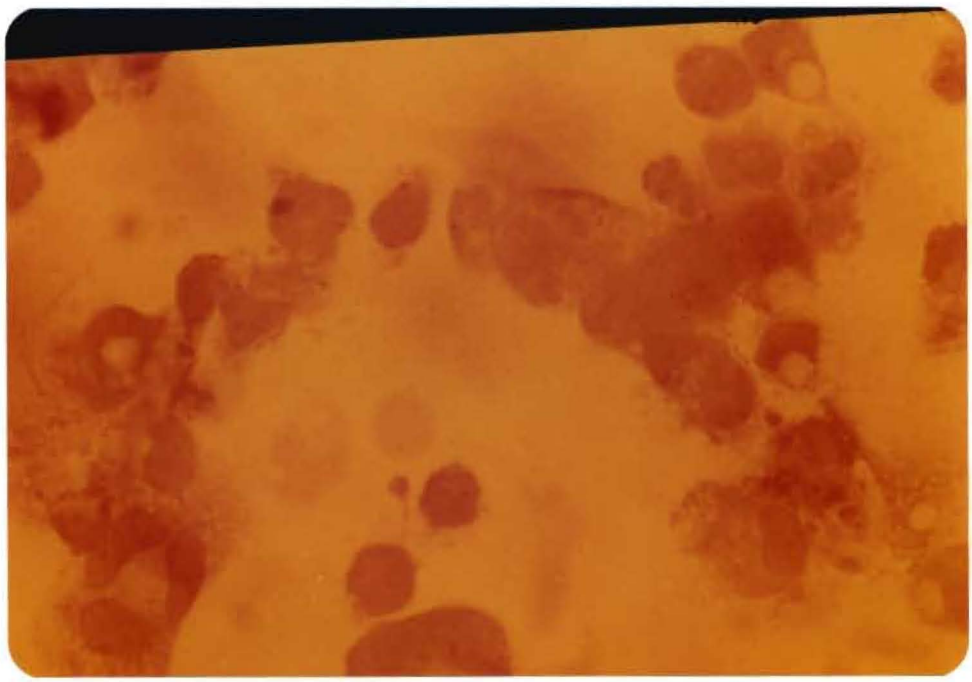
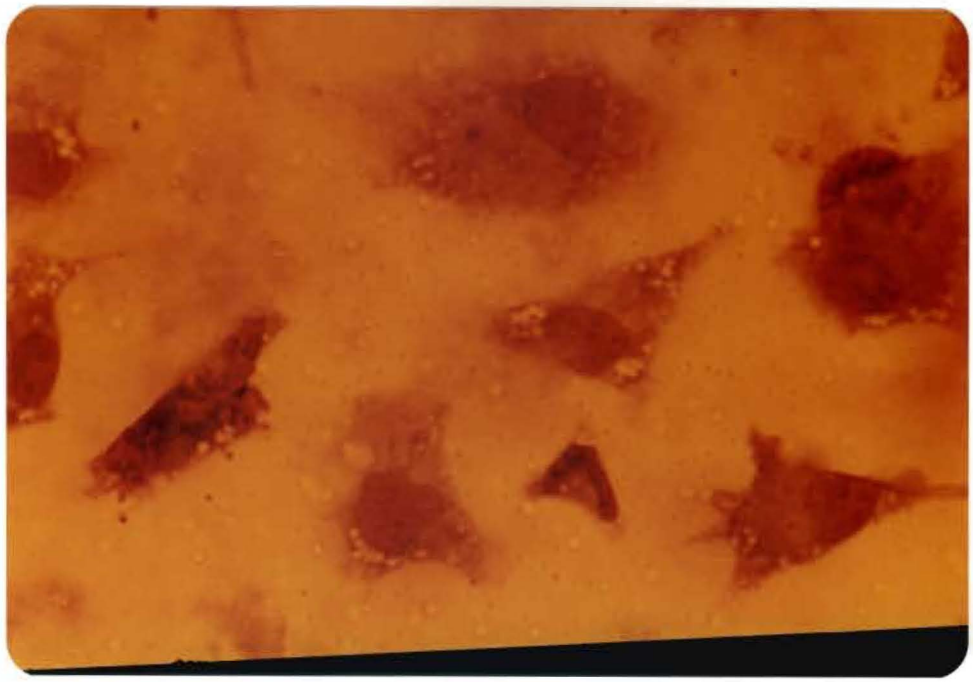
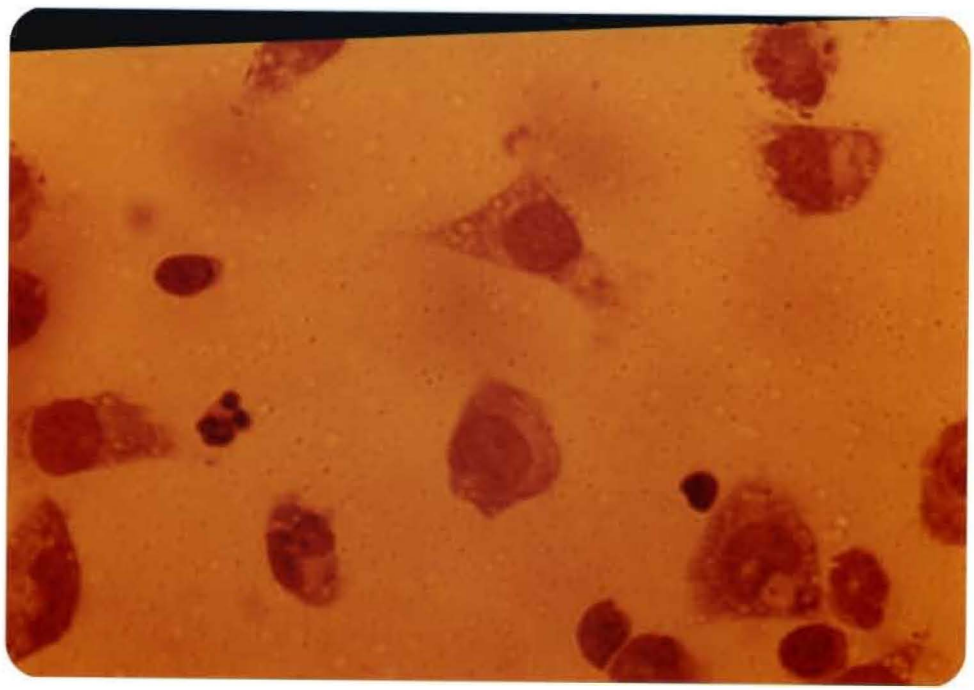


Fig. 5. Uninfected thioglycollate-elicited mouse peritoneal macrophage after twenty-four hours incubation. (X 400) May Grünwald-Giemsa Stain.

Fig. 6. Vaccinia virus infected thioglycollate-elicited mouse macrophages two hours post-infection. No viral induced CPE is exhibited. (X 400) May Grünwald-Giemsa Stain.





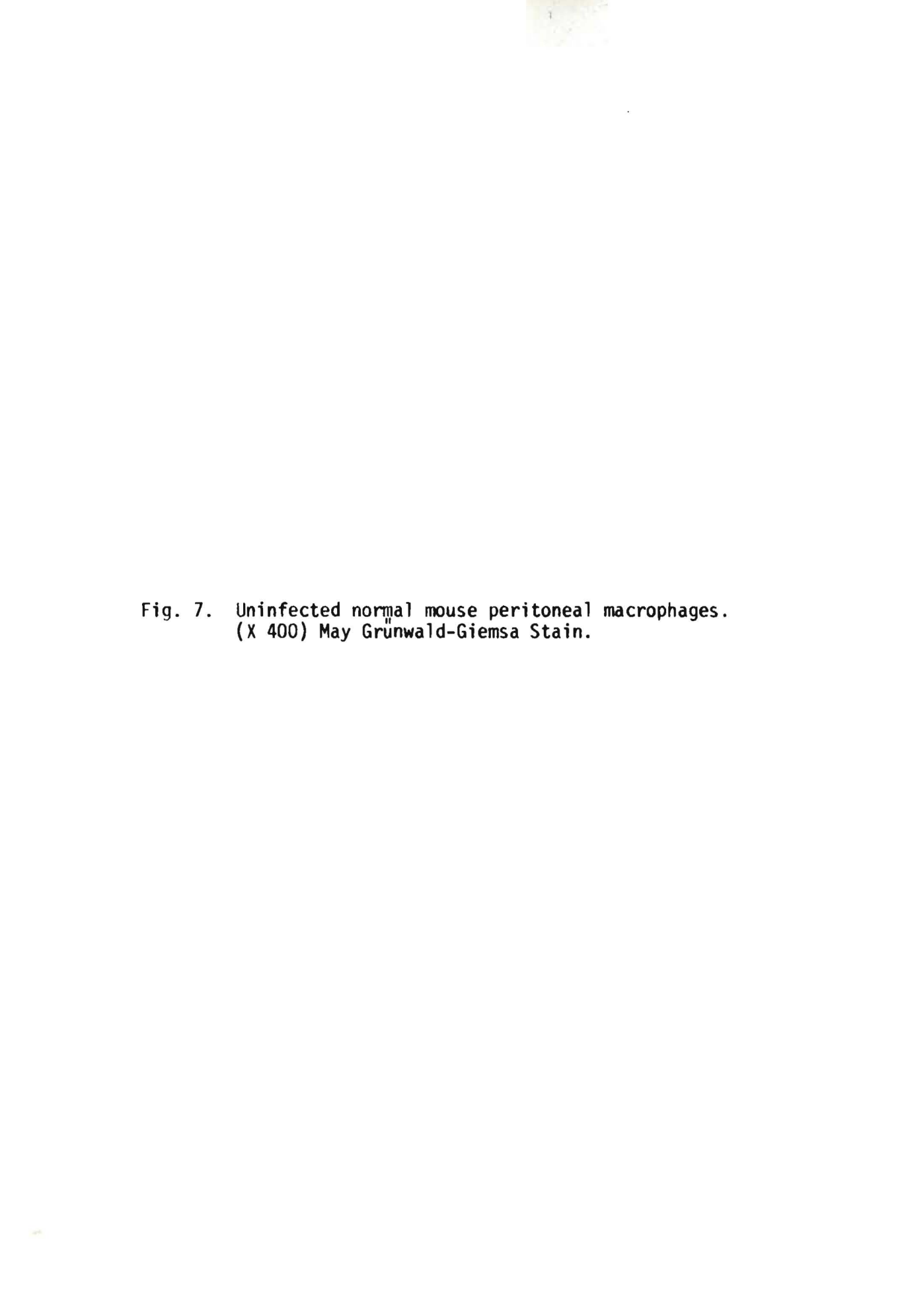
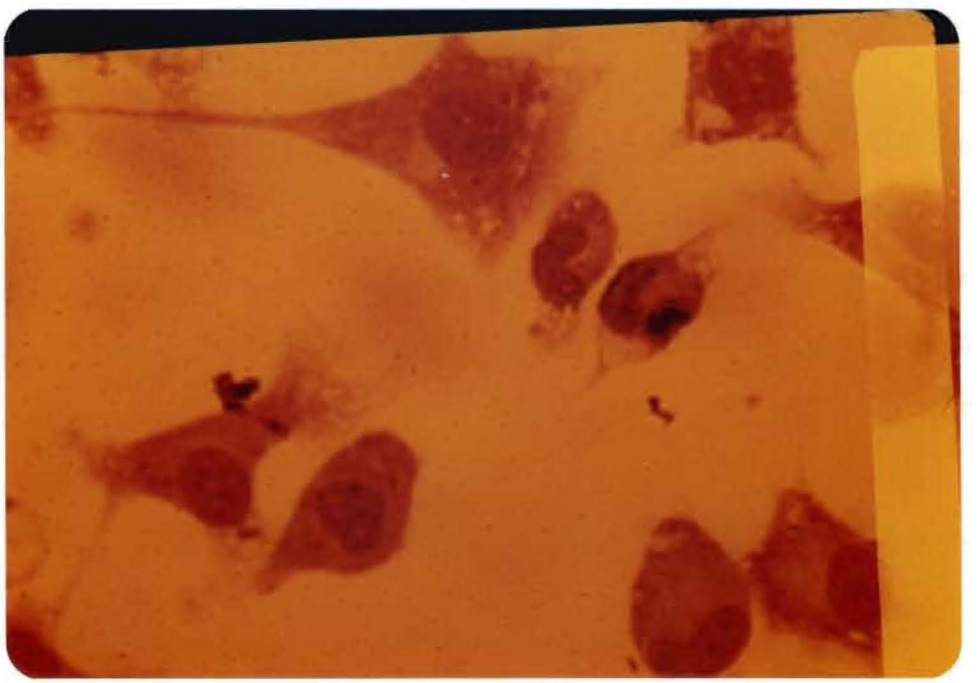


Fig. 7. Uninfected normal mouse peritoneal macrophages. (X 400) May Grünwald-Giemsa Stain.



The macrophages showed no cytopathic effects at any time after infection with vaccinia, and appeared similar to the control macrophages (Fig. 8, 9, and 10).

Electron Microscope Observations

Greer, et al (1973) reported no significant difference between the amount of virus phagocytized by immune rabbit peritoneal macrophages and by normal rabbit peritoneal macrophages. A similar comparison was made in this study with normal rabbit macrophages, thioglycollate-elicited and normal mouse macrophages (Fig. 11). By six hours after infection with vaccinia virus the rabbit macrophage systems contained 50 % more virus within the phagocytic vacuole than was present in either of the mouse macrophage systems. This would indicate the mouse macrophage systems destroyed the virus within the vacuoles, possibley through intracellular digestion.

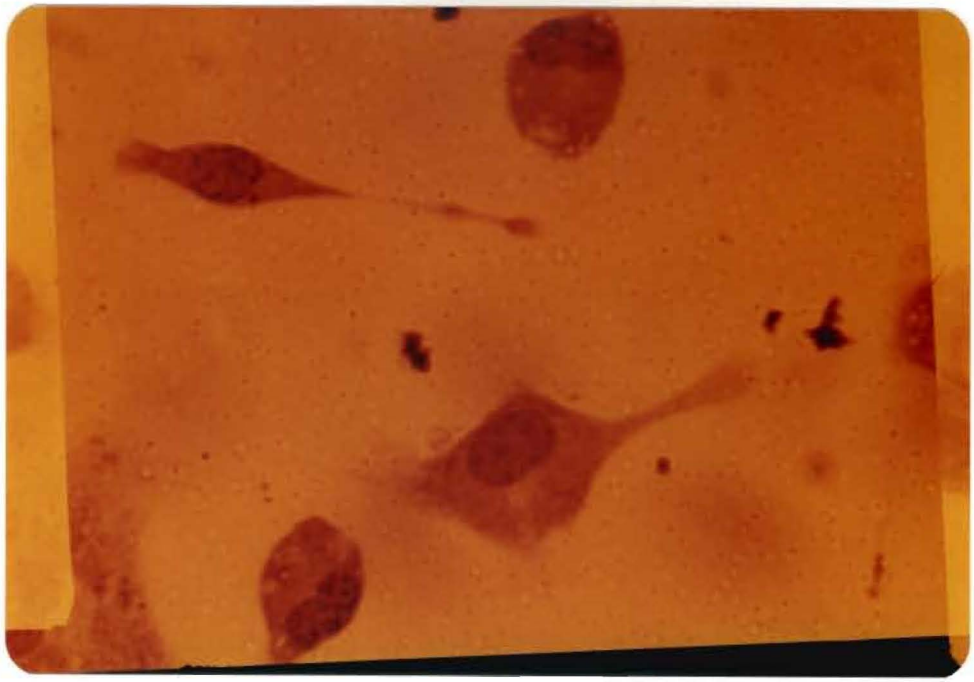
The electron microscope was further utilized to determine whether or not there was any difference in the amount of cytoplasmic core formation between the three macrophage systems (Fig. 12). By two hours after infection with vaccinia virus the rabbit macrophage system contained 50 % more cytoplasmic cores than was present in the thioglycollate-elicited mouse macrophage system and 100 % more than the normal macrophage system. This would indicate that the mouse macrophage systems inhibit viral uncoating. It is suggested that this inhibition is due to the presence of high levels of cellular enzymes.

(1) Electron Microscope Observation of Infected Mouse Macrophages

All macrophages were infected with eight PFU/cell and were found to produce sufficient infected sections for electron microscopic examination. The sections were examined at two, four and six hours post-

Fig. 8. Normal mouse macrophages infected for two hours with vaccinia virus. No viral CPE exhibited. (X 400) May Grünwald-Giemsa Stain.

Fig. 9. Normal mouse macrophages infected for four hours with vaccinia virus. No viral induced CPE observed (X 400) May Grünwald-Giemsa Stain.



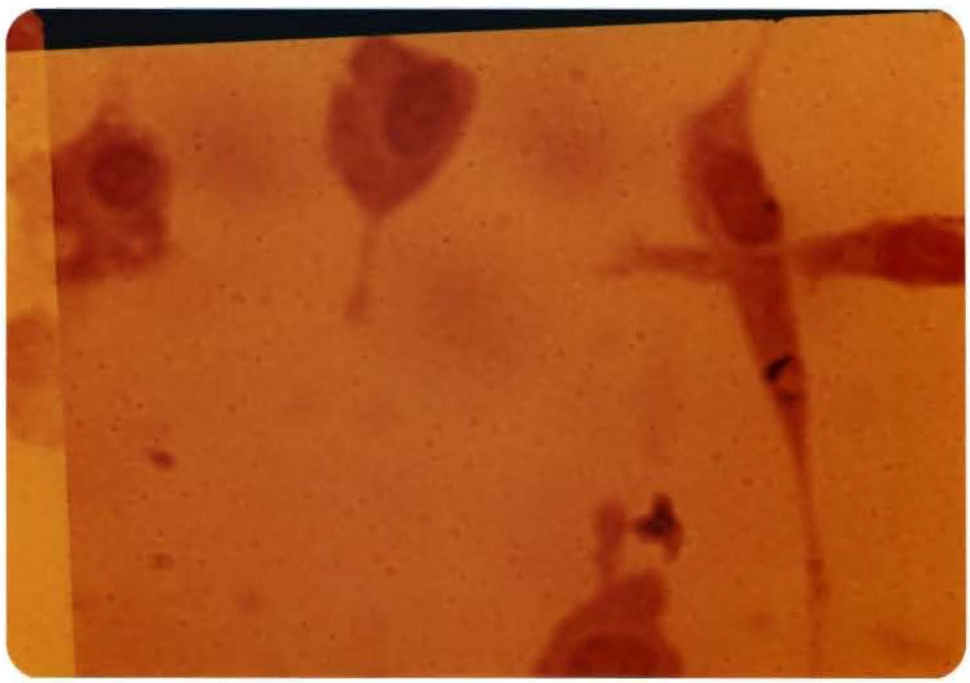


Fig. 10. Normal mouse macrophages infected with vaccinia virus for six hours. No viral CPE exhibited. (X 400) May Grunwald-Giemsa Stain.

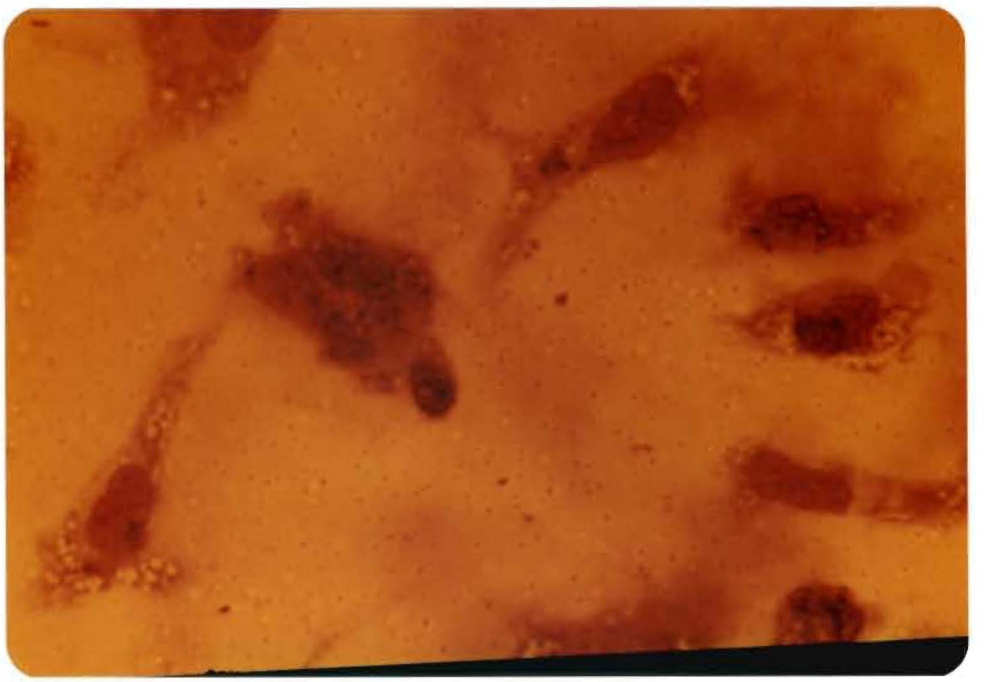


Fig. 11. The phagocytosis of vaccinia virus by three macrophage systems.

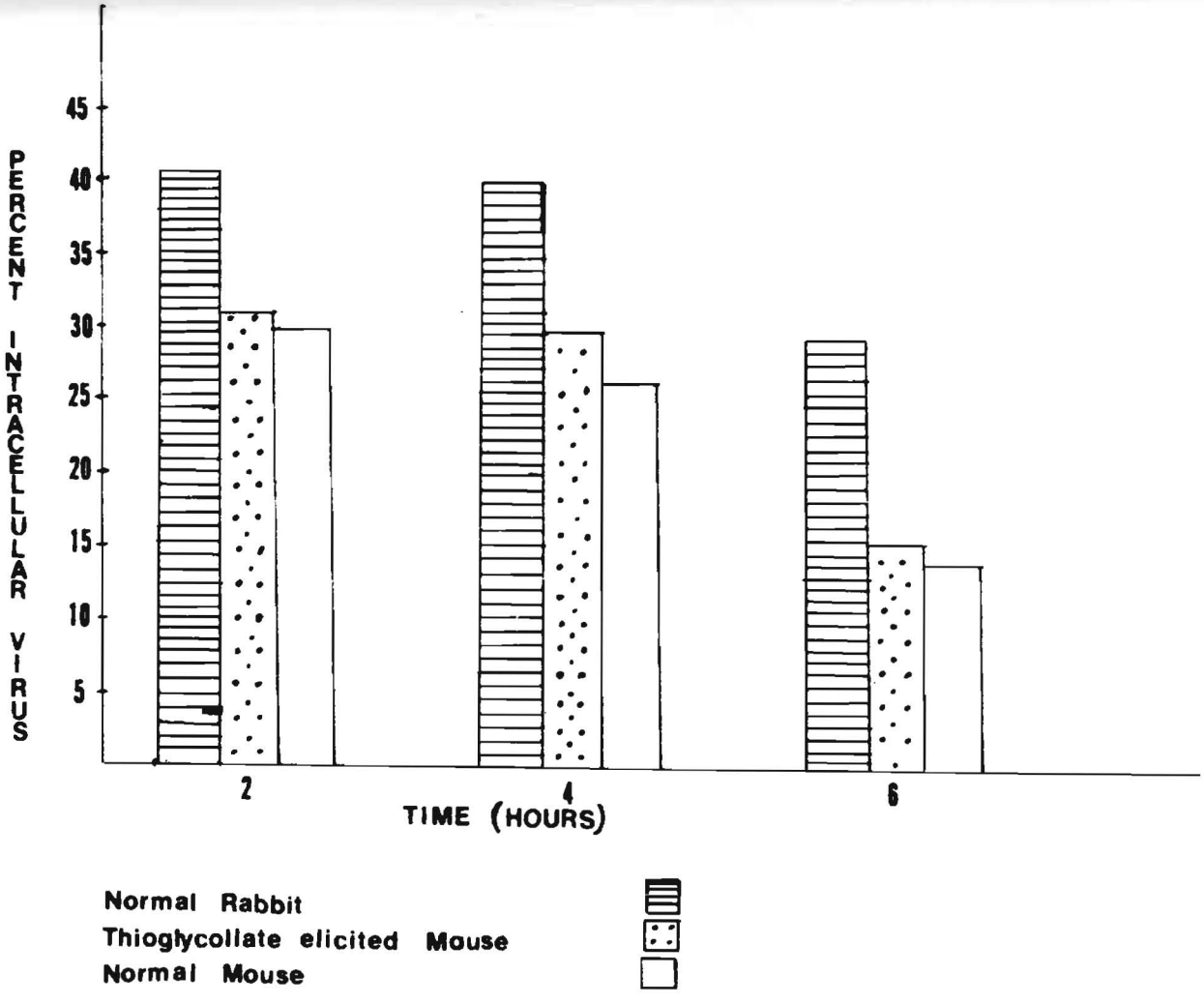
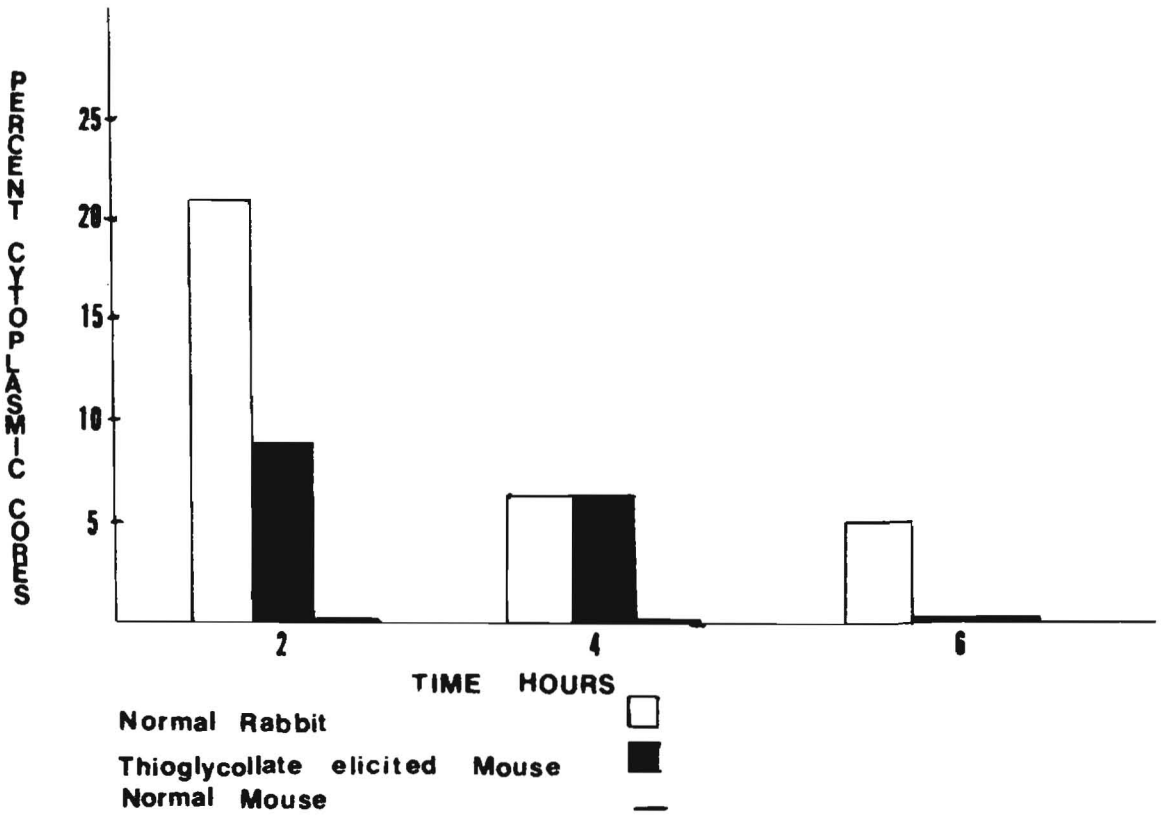

PERCENT INTRACELLULAR VIRUS
45
40
35
30
25
20
15
10
5
2
4
6
TIME (HOURS)
Normal Rabbit
Thioglycollate elicited Mouse
Normal Mouse

Fig. 12. The production of cytoplasmic cores by three macrophage systems.


PERCENT CYTOPLASMIC COBES
25
20
15
10
5
2
4
6
TIME HOURS
Normal Rabbit
Thioglycollate elicited Mouse
Normal Mouse

infection. Two hours after infection, virus was present extracellularly in both thioglycollate-elicited (Fig. 13) and normal mouse macrophages (Fig. 14); intracellular virus was also present in both thioglycollate-elicited (Fig. 15) and normal mouse macrophages (Fig. 16). The virus at this time exhibited the typical viral morphology with a distinct outer coat and a dark staining dumbbell shaped inner core. Observations at four and six hours after infection showed virus still present within the vacuole.

Silverstein (1975) reported viral core formation within thioglycollate-elicited mouse macrophages. However, Ward (1980) could find no cores in the elicited mouse macrophage system. In this study cytoplasmic cores were present in approximately eight percent of the thioglycollate-elicited macrophages examined after two hours infection with vaccinia virus (Fig. 17). These results were in agreement with those of Silverstein (1975). No normal mouse macrophages were found to contain cytoplasmic cores. However, by six hours after infection most virus present inside vacuoles of both thioglycollate-elicited and normal mouse macrophages displayed a slightly diffuse morphology (Fig. 18 and 19). This was interpreted that enzymes within the vacuole were causing destruction of the virus.

(2) Observation of Normal Rabbit Macrophages

To confirm that normal rabbit macrophages could elicit observable core formation, normal rabbit macrophages were infected with eight PFU/cell. Vaccinia virus was present within cytoplasmic vacuoles two hours after infection (Fig. 20). The virus was lying with the outer coat adjacent to the inner membrane of the vacuole and in some instances appeared to be fused with the vacuole. Virus could be found within

Fig. 13. Thioglycollate-elicited mouse macrophages two hours after infection with vaccinia virus. Virus is present outside the macrophage. (X 22000) Uranyl acetate/ lead citrate Stain.

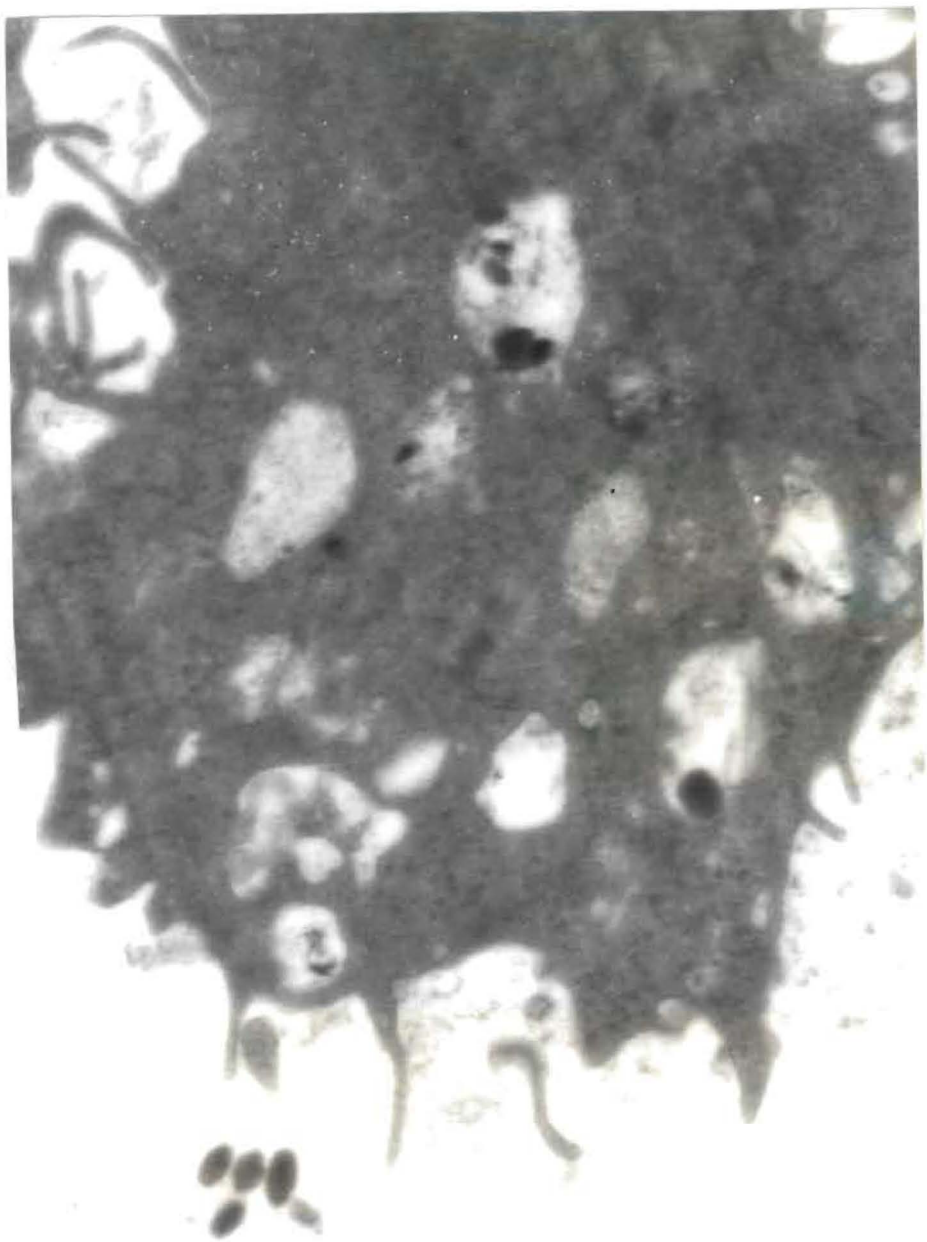


Fig. 14. Vaccinia virus infected normal mouse macrophages. Virus is present extracellularly after two hours infection. (X 26000) Uranyl acetate/lead citrate Stain.

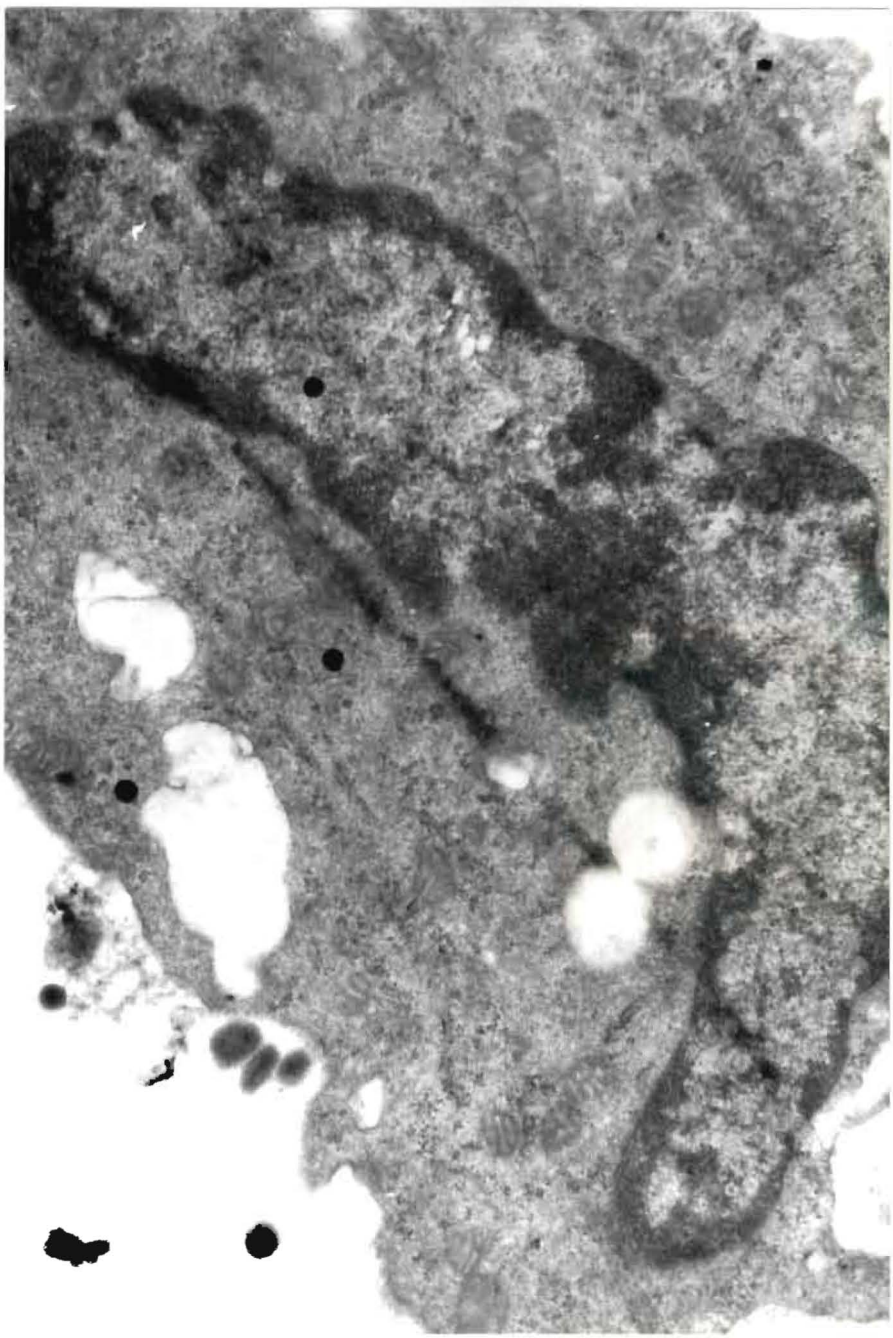


Fig. 15. Vaccinia virus infected thioglycollate-elicited mouse macrophages. Two hours post-infection virus is present within a phagocytic vacuole. (X 30,000) Uranyl acetate/lead citrate stain.

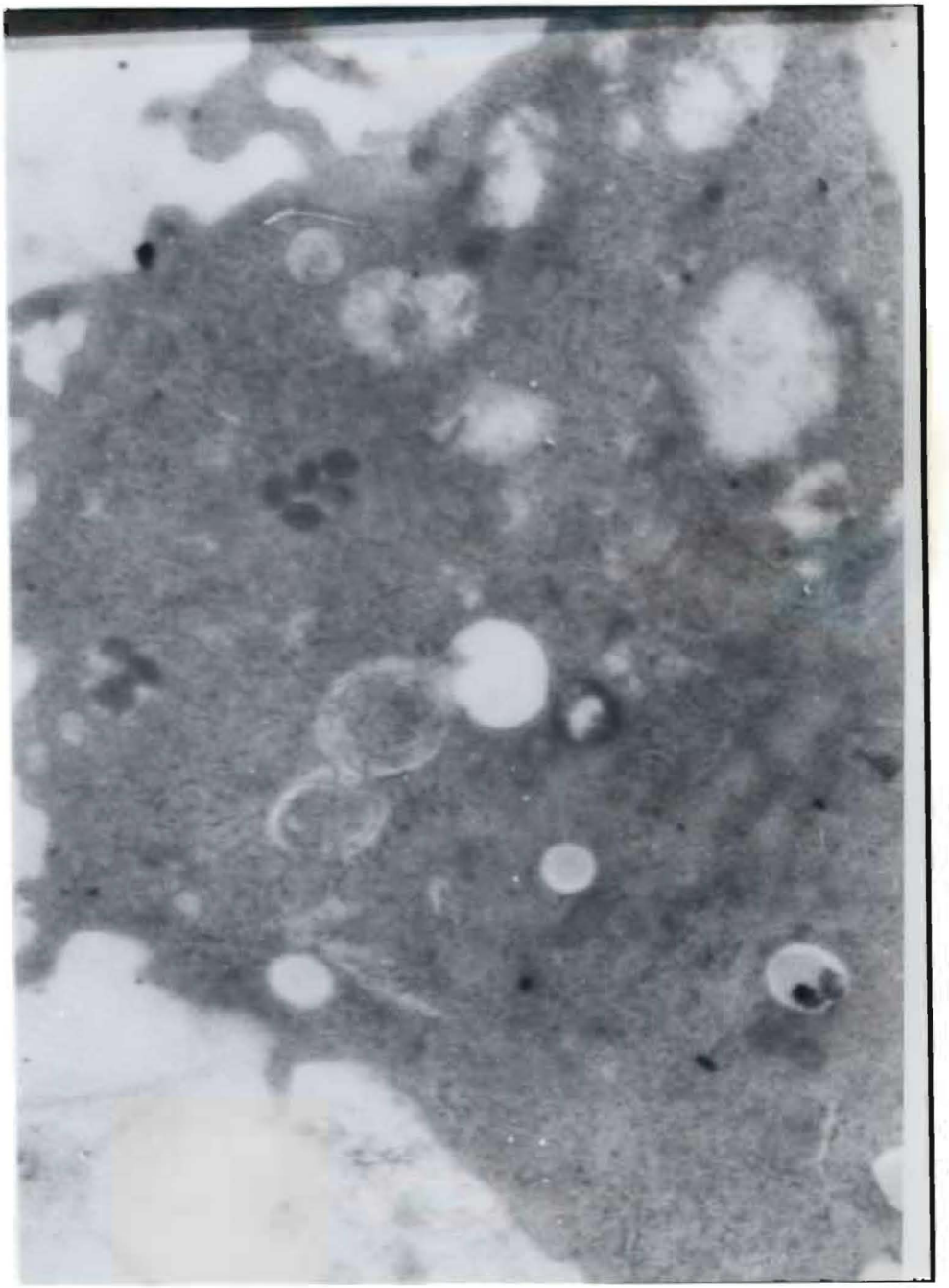


Fig. 16. Vaccinia virus infected normal mouse macrophages for two hours. Virus is present within a phagocytic vacuole. (X 30,000) Uranyl acetate/lead citrate stain.

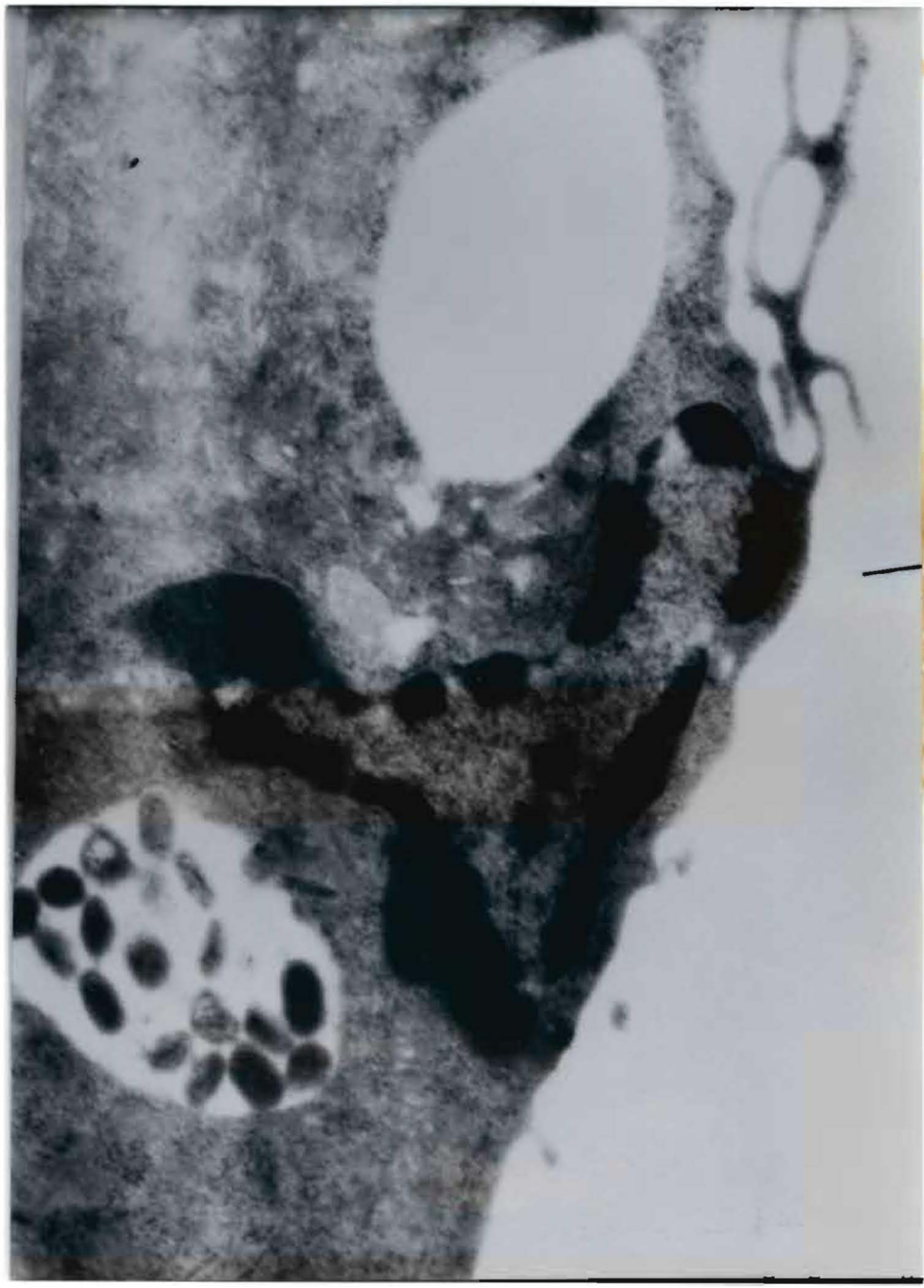


Fig. 17. Thioglycollate-elicited mouse macrophages infected for two hours with vaccinia virus. Cytoplasmic cores are present. (X 35,000) Uranyl acetate/lead citrate stain.

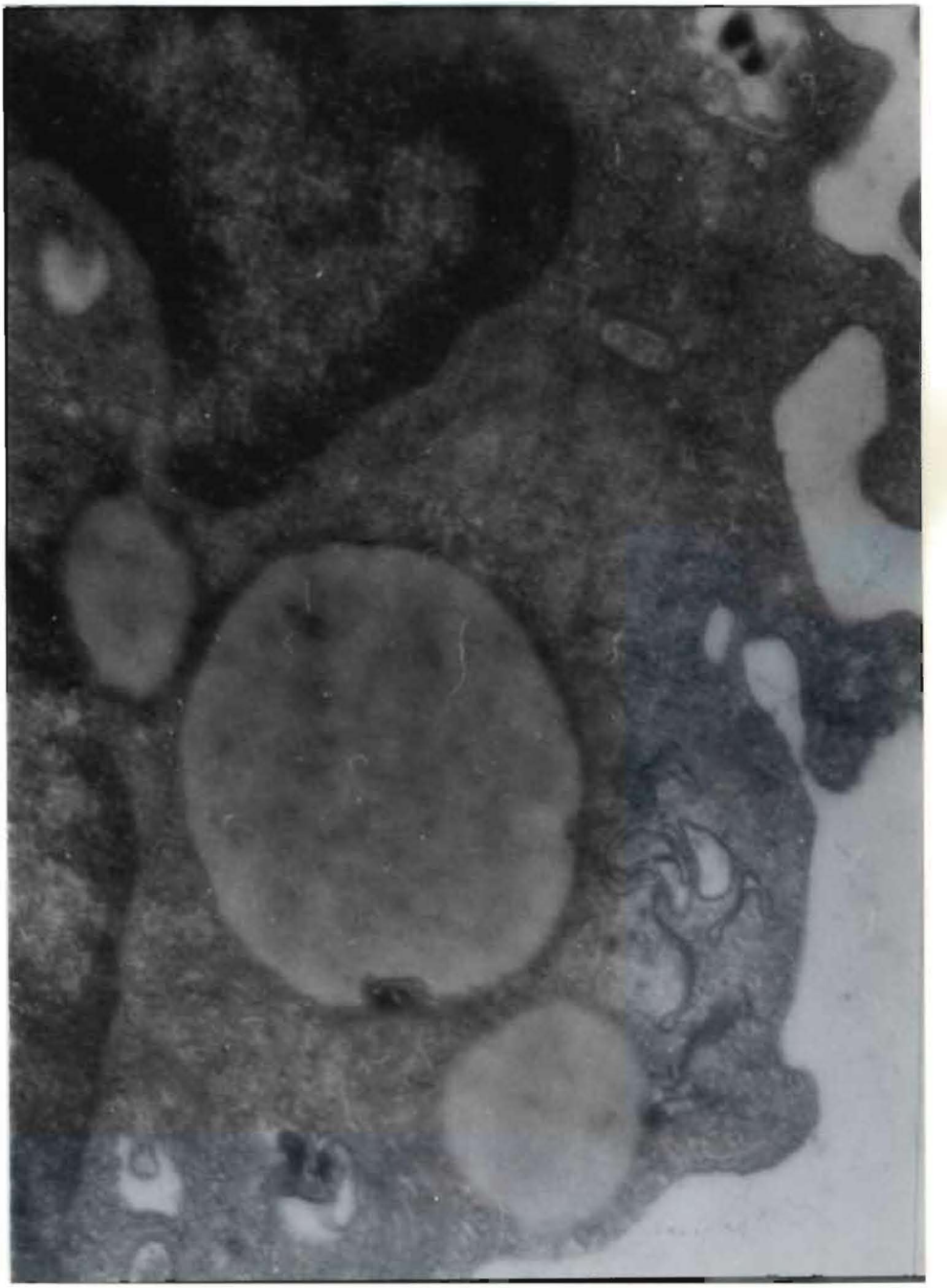


Fig. 18. Thioglycollate-elicited mouse macrophages infected for six hours with vaccinia virus. The virus is inside a phagocytic vacuole and exhibits a slightly diffuse morphology. (X 32,000) Uranyl acetate/lead citrate stain.

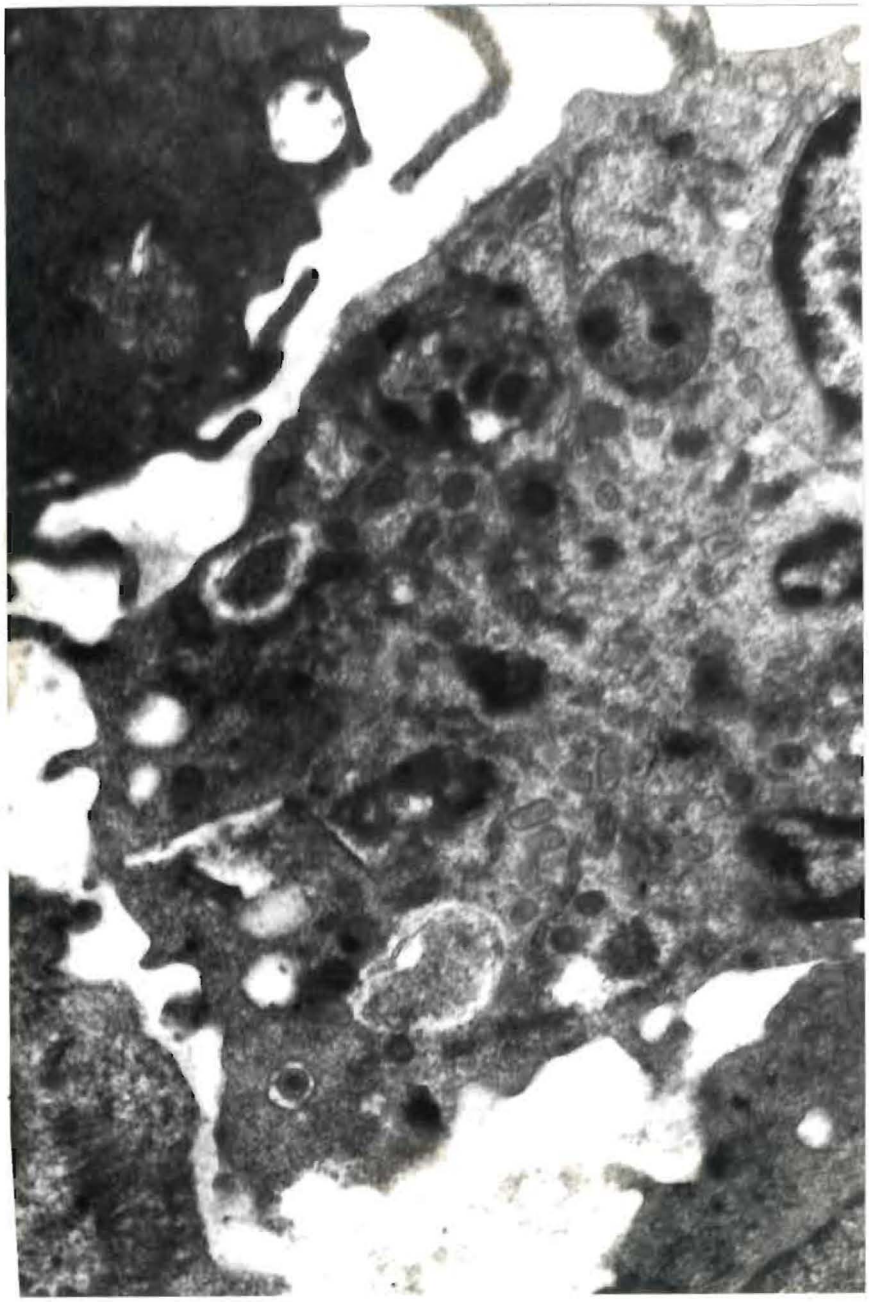


Fig. 19. Normal mouse macrophages infected with vaccinia virus for six hours. The virus within a phagocytic vacuole displays abnormal morphology. (X 30,000) Uranyl acetate/lead citrate stain.

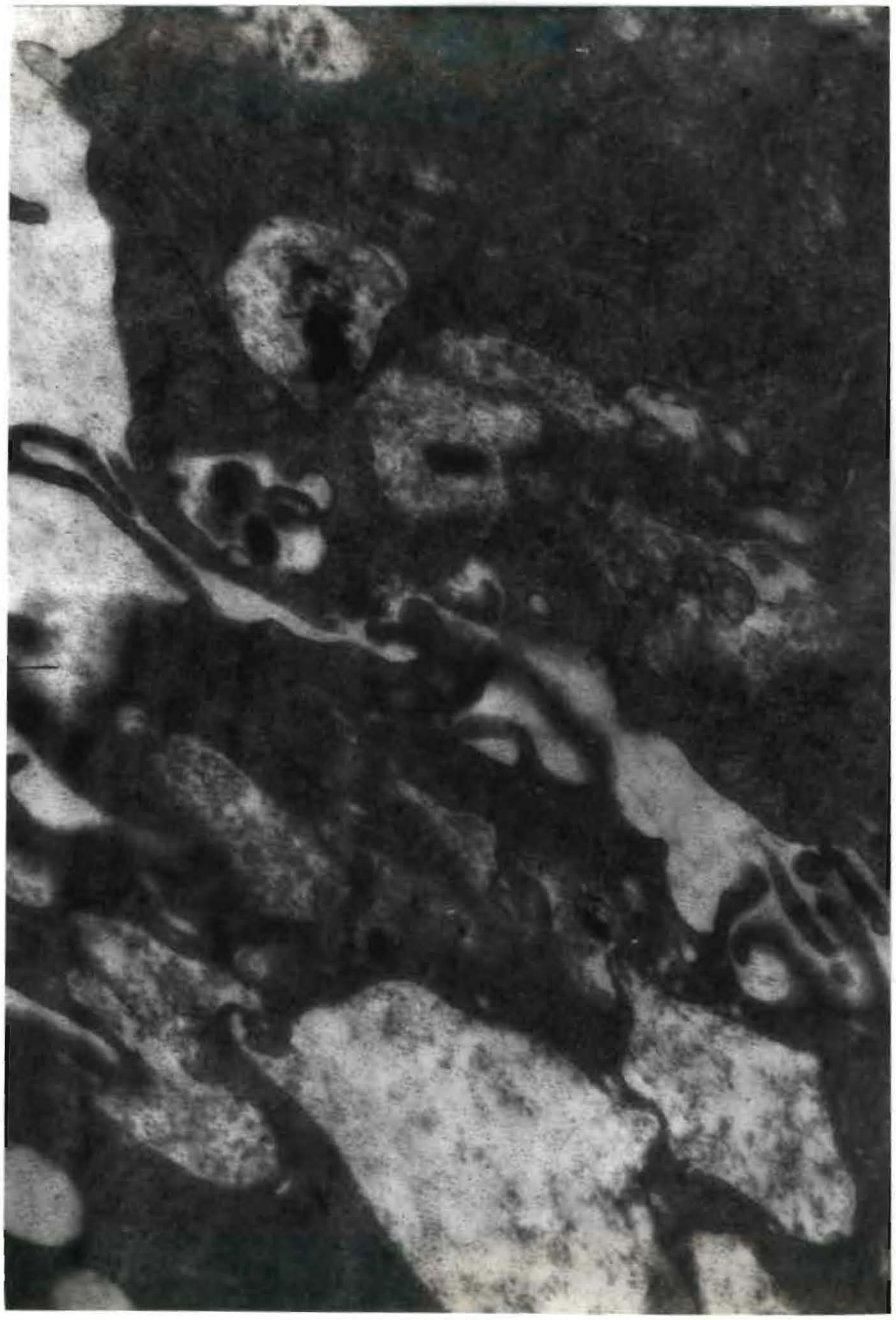
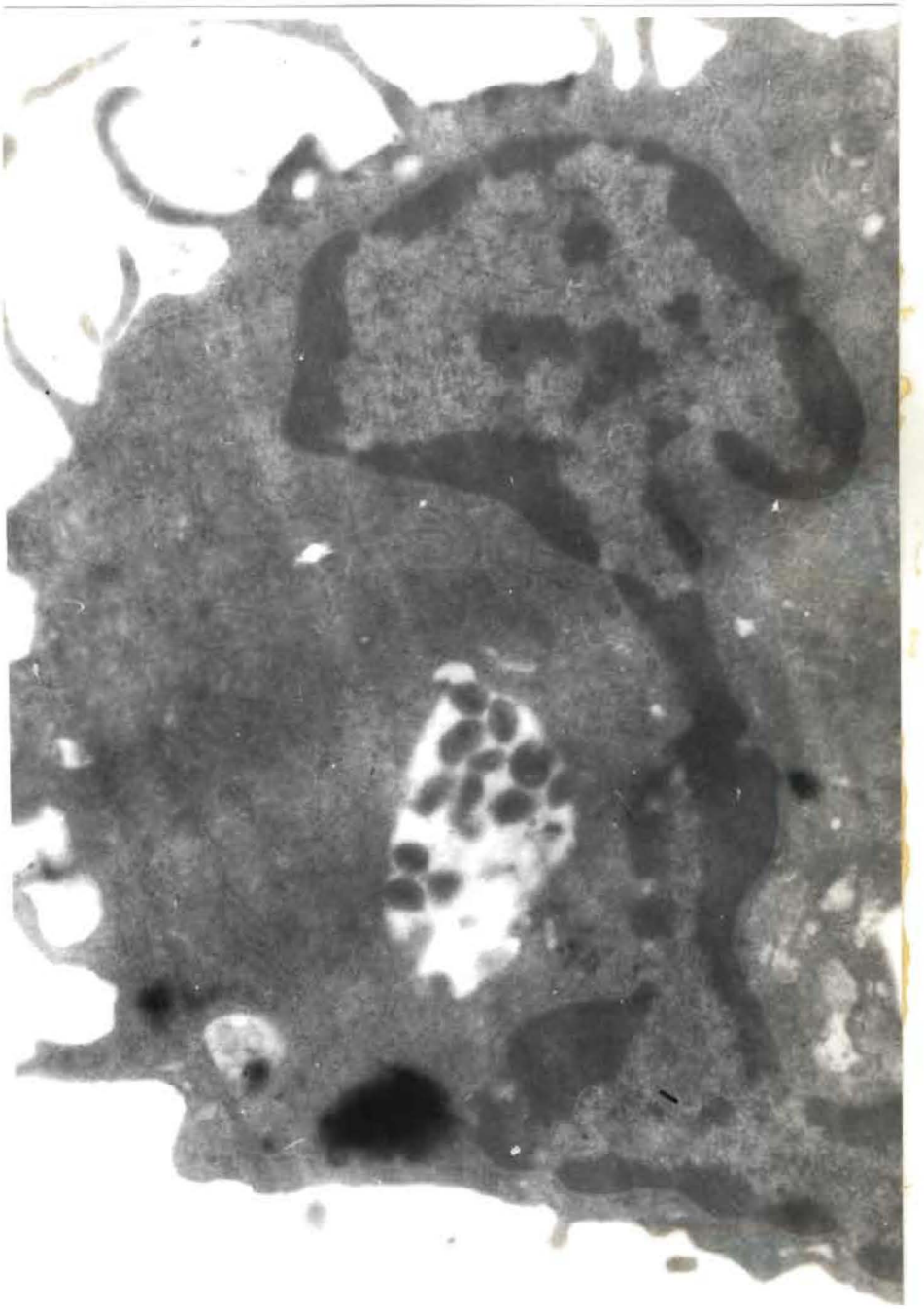
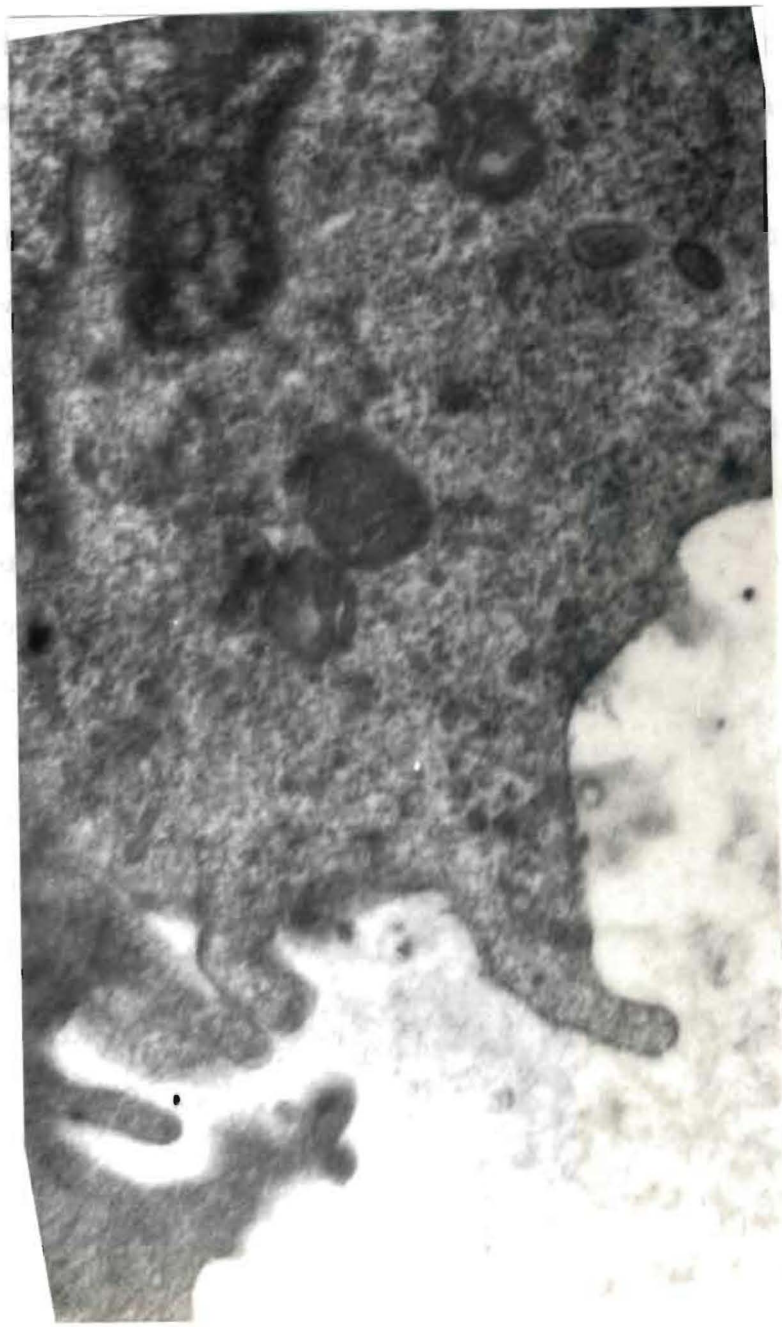


Fig. 20. Normal rabbit macrophages infected for two hours with vaccinia virus. Virus is present within phagocytic vacuole. (X 27,000) Uranyl acetate/lead citrate stain.



vacuoles at all time periods the sections were examined. Twenty-one percent of the normal rabbit macrophages contained cytoplasmic cores within two hours after infection (Fig. 21).

Fig. 21. Normal rabbit macrophages infected with vaccinia virus. Two hours after infection cytoplasmic cores are present. (X 35,000) Uranyl acetate/lead citrate stain.



DISCUSSION

Since Metchnikoff's (1905) description of macrophages and their functions, it has been known that they are capable of phagocytosing many different types of microorganisms. Historically, the interaction of bacteria with macrophages has been the main focus of interest. However, the interaction of viruses with macrophages is now receiving a great deal of attention. Vaccinia virus is a member of the pox virus family. Its interaction with macrophages has been widely studied. The replicative cycle of this virus has been extensively studied with the electron microscope.

The pox viruses are large, DNA containing viruses. The prototype of this group is the vaccinia virus. The typical virion is composed of an outer envelope containing phospholipid and protein. Immediately inside the envelope are two lateral bodies. The central portion of the virus contains double stranded DNA (Joklik, 1966; Briody, 1966). When the virus is observed under the electron microscope, the nuclear material appears biconcave.

During infection of a macrophage the vaccinia virus attaches to the outer cellular membrane (Ginsberg, 1980). The cell extends pseudopods around the virus allowing interiorization. Viral genome replication then follows a two step uncoating process. The first stage of uncoating begins almost immediately after internalization of the virus. The outer envelope of the virus is removed by enzymes which are already present within the phagocytic vacuole (Briody, 1966; Joklik, 1964a; Joklik, 1966; Ginsberg, 1980). There is a lag of one-half to one hour before the second stage of uncoating occurs which results in the liberation of viral DNA.

At the beginning of the second stage of uncoating, when the DNA within the intact core is protected from DNase activity, a DNA-dependent RNA polymerase, which is present in the core, causes transcription of about one-fourth of the viral genome. The transcript is processed within the core, allowing the immediate emergence of early mRNA's, which code for proteins. One of the proteins formed is a proteolytic enzyme which is necessary for final uncoating. Transcription continues for about seven hours after initiation of infection (Ginsberg, 1980).

Normal rabbit peritoneal macrophages allow the replication of vaccinia virus, whereas normal mouse peritoneal macrophages suppress viral replication. Macrophages obtained from mice which have been injected with thioglycollate also suppress viral replication; however, cytoplasmic cores are present which indicates that first-stage uncoating has taken place. The enzyme level, especially that of acid phosphatase, present in each of these macrophage systems may vary depending on the state of activation. It has been shown that acid phosphatase levels are highest in normal mouse peritoneal macrophages (PM) (Schnyder and Baggiolini, 1979) and low in normal rabbit PM (Milligan, 1983). It is suggested that the state of activation of the macrophage may play a role in determining the fate of the virus.

Work done in this laboratory (Milligan, 1983) demonstrated that the level of acid phosphatase in normal mouse PM was higher than in normal rabbit PM. Schnyder and Baggiolini (1979) showed that thioglycollate-elicited mouse PM produced much lower amounts of acid phosphatase than in normal mouse PM. If high acid phosphatase levels are responsible for degradation of vaccinia virus, then it seems likely that normal mouse PM would degrade the virus, whereas thioglycollate-

elicited mouse PM and normal rabbit PM would allow at least first-stage uncoating. It is suggested that an increase in acid phosphatase by normal mouse macrophages causes the destruction of vaccinia virus; lower levels of acid phosphatase in thioglycollate-elicited mouse macrophages and normal rabbit macrophages allow the virus to undergo first stage uncoating.

The function of lysosomes in intracellular digestion of foreign organisms has been well documented. Allison and Sandelin (1963) studied lysosomal enzymes in virus-infected cells in relationship to cytopathic effects. They demonstrated an increase in lysosomal enzymes, both biochemically and histochemically, before cell damage was evident. Allison and Mallucci (1965) studied a variety of viral infections in mouse macrophages. They demonstrated increased acid phosphatase activity in mouse hepatitis virus-type 3 infection and in vaccinia virus infection. Furthermore, they demonstrated a two stage process of lysosomal enzyme activation. The first stage showed an increased permeability of lysosomal membrane which was demonstrable with specific stains for acid phosphatase. The second stage was the release of lysosomal enzymes into the surrounding cytoplasm.

Dales and Kajoika (1964), working with vaccinia virus in Earle's L cells showed that virus, inactivated with antibody, upon interiorization into the phagocytic vacuole was gradually destroyed by enzymes present within the vacuole. This indicates that some undenatured or unblocked component of the viral envelope, probably proteinaceous in nature, is required in order to allow first-stage uncoating to proceed. Dales (1965), also showed that heat denatured vaccinia was degraded within the phagocytic vacuole. He was able to demonstrate the presence

of acid phosphatase in virus containing vacuoles. This led him to suggest that acid phosphatase may participate in the digestion of vaccinia. Although it appears that lysosomal enzymes play a major role in digestion of vaccinia virus in normal mouse PM, other mechanisms cannot be ruled out. Interferon, when discovered by Isaacs and Lindenmann (Burke, 1981), was so named because it appeared to be the agent of viral interference. Cells infected with a virus produced a low molecular weight protein that was secreted into the surrounding medium, so that when the culture media was placed on non-infected cells then challenged with the virus the cells were protected against the virus.

Glasgow and Habel (1962) studied the role of interferon in vaccinia virus infected mouse embryo cells. They determined that the production of interferon was responsible for partial resistance of the mouse embryo cells to the characteristic cytopathic effects seen in vaccinia infected cells. Additional work by Glasgow and Habel (1963) indicated that mice injected with ultraviolet light inactivated vaccinia, then challenged with vesticular stomatitis virus (VSV), were able to inhibit multiplication of VSV. They suggested that leukocytes produced interferon, in response to the inactivated vaccinia, which in turn contributed to the attenuation of the course of VSV infection.

Work done previously in this laboratory suggests that interferon production by vaccinia infected mouse macrophages is not of major importance in preventing the replication of vaccinia virus. Milligan (1983) treated normal mouse PM with cortisone in vitro before infecting with vaccinia virus. Macrophages treated with cortisone suppressed vaccinia replication as well as untreated macrophages. Since cortisone is known to inhibit interferon production (Fenner, et al, 1974), it appears that

interferon production by macrophages is not of primary importance in suppression of vaccinia replication.

In conclusion, the results of this study suggest that the state of cellular activation as well as the level of lysosomal enzymes, expecially acid phosphatase, control the fate of vaccinia virus in the three macrophage systems under observation. Further studies concerning the function of acid phosphatase and other lysosomal enzymes in the control of vaccinia virus infection of peritoneal macrophages should be pursued.

SUMMARY

1. Electron microscope results from vaccinia virus infected peritoneal macrophages indicate that normal rabbit macrophages and thioglycollate-elicited mouse macrophages allow cytoplasmic core formation while no cores are found in normal mouse macrophages.
2. Thioglycollate-elicited mouse macrophages and normal mouse macrophages appear to degrade vaccinia virus within the phagocytic vacuole by six hours after infection.

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