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Title: A Proposed Mechanism for the Inhibition of Vaccinia Virus by Mouse Macrophages

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An investigation of the mechanism of resistance expressed by normal mouse macrophages against IHD vaccinia virus infection was conducted. Results from virus growth assays seemed to show that vaccinia virus is incapable of multiplying in unstimulated mouse macrophages and instead is degraded by them. Autoradiography experiments determined that no significant viral DNA synthesis occurred. This indicated that the mouse macrophage inhibited viral infection at an early stage of virus-macrophage interaction. Results from virus growth assays involving peritoneal macrophages from immature mice indicated that this natural resistance is expressed prior to the fourth week of life.

Incubation of vaccinia virus in susceptible rabbit macrophage or resistant mouse macrophage lysates resulted in a slight drop in virus titer. Because no dramatic viral destruction was observed, it appeared that only the intact cell is responsible in some way for the expression of resistance or susceptibility to vaccinia infection.

The role of lysosomal activation in mouse macrophage resistance to vaccinia was investigated. Acid phosphatase staining techniques revealed the activation of lysosomes in vaccinia infected mouse but not rabbit macrophages. It is suggested that the ability of mouse macrophage lysosomes to activate may play a role in the natural resistance to vaccinia.

# A PROPOSED MECHANISM FOR THE INHIBITION OF VACCINIA VIRUS BY MOUSE MACROPHAGES

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# INTRODUCTION

Animals are subjected to a vast array of potentially harmful microorganisms in every contact with their environment. Obviously, the animal must have a vast array of defenses and barriers to protect itself against infection. It is customary to describe these protective mechanisms by classifying them as an acquired immunity or as an innate immunity to the offending organisms.

Acquired immunity is the term applied to mechanisms which come about as a result of a chance infection or intentional immunization. Acquired protective mechanisms develop shortly after exposure and are specific for the infectious agent. Immunocompetent lymphocytes and macrophages recognize the microbe as foreign and react by initiating and effecting the immune response. This response may involve the production of antibodies by B-lymphocyte derived cells, T-lymphocyte mediated cellular immunity, or both. Numerous long-lived memory lymphocytes also develop during the first exposure to the microorganism. These cells are responsible for a more rapid and intense immune response on subsequent exposure to the microbe.

Innate immunity is the natural ability of an animal to resist infection due to some physiological mechanism or an anatomical barrier. It may be thought of as a nonspecific barrier which is normally present and effective against a great variety of infectious agents (Barrett, 1978). These natural defenses may deter infection by interfering with the invasion, establishment, and multiplication of the microbes and viruses. Innate immunity is probably the main line of defense during the first few days of infection. When these mechanisms fail, an active infection will then result. A final recovery is

dependent on the success of a primary specific immune response against the microbe (Mogensen, 1979).

Many different examples of natural resistance exist in nature. Sheep, cattle, and other mammals are naturally susceptible to anthrax, whereas birds are resistant. Dogs may develop canine distemper but humans do not contract this disease (Barrett, 1978).

Animals are protected against pathogen entry by effective physical barriers such as intact skin, respiratory mucus, and the flushing action of urine and tears which operate at the portals of entry into the body of the host. However, if an infectious agent successfully invades the body, phagocytic cells in the bloodstream and tissues present a formidable internal nonspecific barrier. Phagocytosis of invading microbes is considered to be one of the most active contributing factor to native resistance (Abramoff and LaVia, 1970).

Metchnikoff (1892) is credited with first recognizing the importance of phagocytic cells in host defense. He promoted the idea of a macrophage-microphage system which linked phagocytic cells of the spleen, bone marrow, lymph nodes, body cavities, and blood stream into a functional group on the basis of their common morphology and function. Following Metchnikoff, attempts have been made through the years to improve on the classification of phagocytic cells. Aschoff (1924) proposed the term reticulo-endothelial system and Thomas (1949) preferred the concept of a reticulo-histiocyte system to describe the various cell types involved in phagocytic host protection.

The classification currently utilized is that of the mononuclear phagocytic system (MPS) which groups together highly phagocytic cells on the basis of their common morphology, function, and kinetics. Cells comprising the MPS include, connective tissue histiocytes, Kupffer

cells, alveolar and peritoneal macrophages, free and fixed macrophages of the spleen and lymph nodes, bone marrow macrophages, osteoclasts, sinusoidal cells, and microglial cells in nervous system tissue (Langevoort et al., 1970).

Although the morphology of mononuclear phagocytes varies somewhat due to organ source or activation state, some generalities can be stated. Ruffled cytoplasm, active phagocytosis, and rapid attachment to glass surfaces are common characteristics of mononuclear phagocytic cells. Non-stimulated cells are nearly spherical with numerous irregular pseudopodia protruding from the cell surface. In mature cells, the nucleus is indented and somewhat kidney shaped with prominent nucleopores. Numerous mitochondria and a well developed rough endoplasmic reticulum and Golgi apparatus are observed in electron micrographs. Other details of ultrastructure include microtubules radiating from centrioles and bundles of microfibrils which may be contractile in nature. Many vacuoles and vesicles are present which are thought to represent primary and secondary lysosomes and phagocytic and pinocytic vesicles (Weiss, 1972).

Recent kinetic studies have shown a common ancestory for cells of the MPS. A bone marrow stem cell gives rise to a promonocyte. This cell undergoes mitotic division and differentiation to become a blood monocyte. After a few days in the circulation, monocytes leave the blood stream and become residents in various sites in the body. At this time, further maturation occurs and the cell is thereafter referred to as a functional tissue macrophage (Langevoort et al., 1970).

Although the functions of MPS cells are not yet completely known or

understood, some of the better known functions include clearing blood, lymph, and tissues of infectious agents and effete cells, tumor surveillance, tissue hygiene, and wound healing (Werb, 1982).

The phagocytic and digestive activities of these cells clearly implicate their importance in host defense against potential pathogens such as viruses. Evidence supporting the protective role of macrophages against virus infections is both direct and indirect. For example, the viricidal activities of macrophages are credited for the protection of resistant strains of mice against Arbor B virus (Goodman and Koprowski, 1962). Macrophage depletion by antimacrophage serum injections has been shown to enhance infections of yellow fever virus (Panijel and Cayeux, 1968), vesicular stomatitis virus (Hirsch et al., 1969), and herpes simplex virus (HSV) (Zisman et al., 1970) in mice. Silicon dioxide which is specifically toxic for macrophages has also been used to demonstrate the role of macrophages as an early barrier to HSV infection in mice (Zisman et al., 1970).

Studies utilizing variable host susceptibility to virus infection due to virus strain have yielded valuable information on macrophage-virus interactions. In some cases, the ability of a virus strain to replicate in macrophages has been correlated with viral virulence for the whole animal (Allison, 1974). For example, Bang and Warwick (1957) infected chicken cells with a virulent and an avirulent strain of Newcastle disease virus. Both strains grew equally well in fibroblasts. Macrophages were shown to resist the avirulent strain but succumbed to the virulent strain. Likewise, a study by Roberts (1963) utilizing virulent and avirulent ectromelia virus strains correlated virulence with the ability of virus to infect Kupffer cells. Avirulent virus, although capable of infecting parenchymal cells, did not infect or

replicate in Kupffer cells to the same extent as the virulent strain.

Johnson (1964), studying the age dependent susceptibility of mice to HSV encephalitis, showed that the infection and uptake of virus by adult and suckling mouse macrophages were similar. However, suckling mouse macrophages tended to allow infection of surrounding cells whereas adult macrophages did not. He proposed that a "barrier" to HSV encephalitis existed in adult mice and that this barrier was due to the activity of macrophages.

Hirsch et al. (1970), in an extension of these studies, found that transplanting large numbers of stimulated adult macrophages into syngenic suckling mice protected them from infections of HSV. According to his studies the adult macrophage was more efficient at phagocytizing and destroying the virus than the immature macrophage.

Excellent studies involving susceptibility of various strains of mice to a particular virus have established the importance of genetics in natural resistance. Early studies by Theis and Koprowski (1961) revealed a cellular basis for the inherited resistance of certain strains of mice to West Nile virus. It was shown that kidney cells of both susceptible and resistant strains of mice supported virus growth. However, West Nile virus replicated in spleen cells and peritoneal macrophages of susceptible mice but not in macrophages from resistant animals. The inherited difference in susceptibility seemed to be due to a difference in the ability of the macrophage to destroy the virus.

Similar results were obtained in studies utilizing a mouse hepatitis virus (MHV-2) model. Bang and Warwick (1960) reported in studies later confirmed by Kantoch et al. (1963) that $C_3H$ strain mice were resistant and PRI strain mice were susceptible to infection with MHV-2. In vitro cultures of macrophages from these inbred strains mimicked the

susceptibility or resistance in vivo. Moreover, by making the appropriate crosses and backcrosses between the two strains, it was shown that the susceptibility to infection was inherited as a single dominant gene. It was suggested that this gene is expressed phenotypically at the level of the macrophage.

Lindenmann et al. (1978) have shown in a similar series of experiments that resistance of mice to a mouse macrophage adapted strain of influenza virus is inherited as a single gene. Techniques allowing the removal of peritoneal macrophages without animal sacrifice enabled them to show that resistance of the whole animal and resistance of peritoneal macrophages in vitro were manifestations of the same phenomenon.

Although the role of macrophages in natural resistance to viruses is well documented, the exact antiviral mechanisms remain in question. Much work has been done on virus-macrophage interactions by employing various models. Because vaccinia virus has been highly characterized in terms of its morphology, antigenicity, and multiplication cycle, it has been utilized to study cell-mediated immunity and host resistance in normal and immune animals.

Differences in species susceptibility to vaccinia virus have been noted. Vaccinia virus causes a non-lethal infection in rabbits, but has no apparent effect on mice unless given intravenously in large doses (Silverstein, 1970). In vitro studies by Beard and Rous (1938) showed that normal rabbit macrophages were incapable of inhibiting vaccinia replication. Tompkins et al. (1970) showed that macrophages from rabbits immunized with vaccinia virus were capable of resisting vaccinia infection. This inhibition has been attributed to the development of activated macrophages which were specifically stimulated

by immune lymphocyte products. Buchmeier et al. (1979) employed uncoating and DNA synthesis assays, immunofluorescence, immunodiffusion and electron microscopy to study the inhibitory mechanisms inherent in the immune macrophage. She found that the immune macrophage aborted the vaccinia infection late in reproductive cycle.

Conflicting results have been reported concerning the interaction of vaccinia virus with normal mouse macrophages. Nishmi and Niesikowski (1963) showed that vaccinia virus could replicate in renal epithelium and lung fibroblasts from suckling mice, but not in macrophages. Glasglow (1965) examined the role of macrophages in protecting mouse embryo fibroblast cultures from vaccinia infection. He suggested this protective effect may be due to macrophages slowing the spread of infection or eliminating the virus.

Mims (1964) injected mice with either virulent CL-R strain or avirulent CL strain vaccinia and followed the infection by immunofluorescent microscopy of liver sections. Virulent CL-R strain grew in normal Kupffer cells and thus acquired access to parenchymal cells. However, CL virus infection was inhibited by Kupffer cells.

The results of Koszinowski et al. (1975) are contrary to those of Nishmi and Nisikowski in that the WR strain of vaccinia was found to replicate in peritoneal resident cells of control mice, but not in those cells of vaccinia immune mice. Studies by Ueda and Nozima (1973) revealed that the Lister strain of vaccinia could infect and multiply in mouse macrophages but the titer produced was consistently low.

Studies in this laboratory involving vaccinia virus - mouse models have shown the IHD strain of vaccinia virus to be incapable of replicating in mouse macrophage cultures (Schultz, 1966; Ward, 1980).

Schultz (1966), utilizing fluorescent antibody and acid phosphatase stains produced evidence that the antiviral activity of the macrophages may be connected with the state of lysosomal activation. Ward employed autoradiography, an uncoating assay, and electron microscopy in an attempt to determine the stage of the virus replication cycle in which the infection was terminated in non-immune macrophages. His findings suggest that the viral core is not released into the macrophage cytoplasm as occurs in susceptible cells. Furthermore, electron micrographs of infected mouse macrophages provided evidence that the virus was degraded in the phagosome.

The present study was initiated in an attempt to show that the IHD strain of vaccinia virus did not replicate in mouse macrophages and to further elucidate the antiviral mechanism.

# MATERIALS AND METHODS

## Experimental Animals

Mice were obtained from the Emporia State University Division of Biological Sciences animal facility. New Zealand white rabbits were obtained locally for use in rabbit peritoneal macrophage studies.

## Cell Culture Media

Mouse and rabbit peritoneal macrophages were harvested in Eagle's Minimum Essential Medium (MEM) containing two units of heparin per ml. Mouse embryo fibroblasts were harvested in Dulbecco's phosphate buffered saline (DPBS) with magnesium and calcium ions. All cell types were cultured in MEM, supplemented with either sterile calf serum (Colorado Serum Company) or fetal calf serum (Kansas City Biological) at a final concentration of 5.0 % or 10.0 %, as required. Combiotic (200 units/cc Penicillin G, 100 units/cc Streptomycin) was added to all cell harvest and culture media. Sterile 1.5 % or 75 % sodium bicarbonate solutions were used to adjust the pH of the culture when necessary.

## Cell Culture

(1) Mouse Embryo Fibroblasts

Gravid 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 carefully removed and placed in sterile petri dishes containing DPBS and combiotic. Upon removal from the uterus, the embryos were decapitated and eviscerated. Blood and loose tissue were removed by three successive washes in sterile DPBS. The embryos were minced and placed in a trypsinizing flask containing a sterile Pronase (Calbiochemical, La Jolle, CA) solution (0.125 g Pronase in 50.0 ml

DPBS). The cells were dispersed for two hours at room temperature by gentle spinning on a magnetic stirrer. 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 2.0 X $10^6$ cells per ml with MEM plus 10 % calf serum and combiotic. Cells were then planted in either Roux flasks for virus propagation or plastic tissue culture flasks for virus titrations.

(2) Mouse Peritoneal Macrophages

Adult mice were sacrificed by cervical dislocation and the fur was wetted with disinfectant. The abdominal skin was cut and carefully pulled back. The abdominal muscles were swabbed with disinfectant, and 6.0 ml quantities of MEM plus heparin were injected into the peritoneal cavity using a 25 ga. needle. The abdomen was then gently massaged for approximately one minute to suspend peritoneal resident cells. A longitudinal incision was made in the abdominal muscles and the cell suspension was removed aseptically and deposited in a sterile 125 ml Erlenmeyer flask. Harvests from six to ten mice were pooled.

The cell suspension was washed in MEM plus Combiotic, counted, and adjusted to a concentration of 3.0 to 3.5 X $10^6$ cells per ml. Cells were then planted in flying coverslip tubes in 1.0 ml quantities and incubated 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 1.0 ml quantities of MEM plus combiotic and 10 % calf serum.

(3) Rabbit Peritoneal Macrophages

Five days prior to the harvest, rabbits were injected intraperitoneally with 50 ml sterile mineral oil. For harvest, the rabbits were sacrificed by air embolism. The fur was wetted and the abdominal skin was laid back. Fifty ml of MEM plus heparin and combiotic were injected into the peritoneal cavity. The abdomen was gently massaged for approximately one minute, then aseptically opened and 100 ml of harvest medium was introduced into the cavity via a volumetric pipet. Fluid was withdrawn with a 10 ml pipet and deposited in a separatory funnel. After completion of the harvest, the aqueous and oil phases of the harvest were allowed to separate at 4°C for 15 minutes. The aqueous phase containing cells was pelleted by centrifugation, washed three times in MEM plus combiotic, counted, and cultured as described.

Virus Propagation

The IHD strain of vaccinia virus utilized 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 warm sterile DPBS. Infection was effected by the addition of 15 ml of MEM containing $10^4$ and $10^5$ plaque forming units (PFU) of IHD vaccinia per ml. After a 2.0 hour adsorption period, the infecting medium was removed and the monolayers were again washed twice with DPBS. Fresh MEM plus combiotic and 5.0 % calf serum was added and the cultures were incubated until complete cell destruction was observed. Virus induced cytopathic effect (CPE) was usually evident at 18 to 24 hours after infection and monolayer destruction was normally complete 48 hours after infection.

Harvesting the virus consisted of subjecting the infected monolayer and medium to three cycles of freeze-thaw to release

intracellular virus. Cell debris was removed by centrifugation and the resulting stock virus solution was stored at -70°C.

Virus Assay

Viral titer expressed as plaque forming units (PFU) per ml was obtained by performing plaque assays on mouse embryo fibroblast monolayers. Mouse embryo cells were planted in plastic tissue culture flasks. The cells were twice washed with DPBS just prior to complete monolayer formation and appropriate dilutions of IHD vaccinia in MEM plus 2.0 % calf serum were then added to 1.0 ml amounts to the flasks. A two hour adsorption period followed during which the flasks were gently agitated at 10 minute intervals to ensure random plaque formation. Unadsorbed virus was removed after incubation by washing the cultures twice with DPBS, followed by the addition of fresh MEM plus combiotic and 5.0 % calf serum. The flasks were then incubated for 40 to 48 hours at 37°C until plaque formation was evident. For counting plaque, 1.0 ml of a 10 % aqueous solution of crystal violet was added to each flask. Removal of the stain was followed by washing the monolayer with tap water. Viral-induced plaques appeared as round clear areas distributed in the purple stained monolayer.

Vaccinia Infection of Mouse Peritoneal Macrophages

Peritoneal macrophages were harvested and cultured in flying coverslip tubes as described. Twenty-four hours after planting, the coverslip cultures were washed with MEM plus combiotic to remove cell debris and unattached cells. Infection was initiated by the addition of 1.0 ml of MEM plus 2.0 % fetal calf serum containing approximately 2.0 to 5.0 X $10^6$ PFU/ml vaccinia virus to the cultures. A two hour adsorption period followed in which the tubes were agitated at 10

minute intervals. Following adsorption, the tubes were washed again and fresh MEM plus 10 % fetal calf serum was added. Coverslips were pulled for virus assay and May Grünwald - Giemsa staining was performed at 2.0, 24, 48, and 72 hours after infection. Duplicate coverslip tubes were pulled and frozen at each time interval for virus assay. After three cycles of freeze-thaw, appropriate dilutions of the virus containing medium were plated on mouse embryo fibroblast and assayed for virus titer as described.

Staining

(1) May Grünwald - Giemsa

Coverslips of macrophage monolayers were stained with May Grünwald - Giemsa and examined for viral - induced CPE. After drying, the coverslips were fixed in absolute methanol for five minutes, stained in May Grünwald stain for nine minutes, and in Giemsa for 14 minutes. Coverslips were then rinsed in absolute acetone and a 50:50 acetone-xylene mixture, cleared in xylene for five minutes, and finally mounted cell side down on microscope slides with permount.

(2) Acid Phosphatase Reaction

It was necessary to provide positive and negative staining controls for comparison purposes for each experiment. Therefore, lysosomes were artificially "activated" by fixing one coverslip in cold 10 % neutral formalin. This coverslip was processed simultaneously with an unfixed negative control before the start of each experiment.

All coverslips were incubated at 37°C for two hours in a substrate solution prepared according to the method of Schultz (1966). After incubation, the coverslips were rinsed in physiological saline, followed by a 10 second rinse in 1.0 % glacial acetic acid. The coverslips were then stained in 22 % ammonium sulphide for three minutes, rinsed in

distilled water, and mounted cell side down in phosphate buffered glycerol. The activation of lysosomes was determined by the appearance of discrete, dark staining bodies in the cell cytoplasm.

Carbon Uptake Studies

The phagocytic ability of four week, adult, and cortisone treated macrophages was compared by determining their ability to engulf carbon particles. After 24 hours in culture, the medium was removed and replaced with a 1:1,000 dilution of India Ink in MEM. The coverslips were pulled, fixed, and stained with May Grünwald - Giemsa after a one hour incubation period. The number of cells containing carbon particles per 1,000 cells counted was determined.

Autoradiography

Mouse peritoneal macrophage cultures were infected at a multiplicity of infection of 0.1. MEM containing 1.0 μCi/ml of tritiated thymidine was then added to the infected and control tubes. At intervals of 4.0, 8.0, and 24 hours, coverslips were pulled, rinsed in three changes of Hank's balanced salt solution (HBSS), air dried, and fixed in absolute methanol for five minutes. The coverslips were then mounted cell side up with permount and left to dry for three days.

All subsequent procedures were carried out in a 65°F darkroom. NTB-2 nuclear track emulsion (Eastman Kodak Company) was melted and heated for 30 minutes in a 45°C water bath. The slides were quickly dipped into the emulsion and the excess removed by draining onto a moist paper towel. The emulsion was then dried for 30 minutes under cool air flow. Next, the slides were placed in slide boxes containing Drierite which were wrapped in aluminum foil and stored in a refrigerator at 4.0°C. After a 10 day exposure period, the slides were

developed by processing for 10 minutes in each of the following: D-19 developer (Eastman Kodak Company), water, Fixer (Eastman Kodak Company), and a water bath. The slides were then stained in Giemsa for 20 minutes, followed by a water rinse. Staining of the cytoplasm was performed by dipping the slides in Delafield's Hematoxylin for two minutes followed by 30 seconds in saturated lithium carbonate. The slides were then air dried and covered with a 50 mm X 25 mm coverslip mounted with permount.

The slides were examined microscopically to determine the amount of cytoplasmic labeling per 1,000 cells.

Macrophage Lysate Studies

Peritoneal macrophages were harvested from mice and rabbits as described. The cell suspension of each harvest was adjusted to 1.0 X $10^7$ cells per ml with MEM plus combiotic. Aliquots of cells were subjected to four cycles of freeze-thaw to release lysosomal enzymes, followed by centrifugation at 3,000 RPM for 30 minutes to remove cell debris. Stock virus was added in 0.3 ml amounts to 2.5 ml of cell lysate and to 2.5 ml of MEM as a control. The mixtures were incubated at 37°C for six hours and 0.5 ml samples were removed for virus assay at zero time, 2.0, 4.0, and 6.0 hours. Virus titrations were performed on mouse embryo fibroblasts as described.

Cortisone-21-Phosphate Treatment of Mouse Macrophages

Adult mouse macrophages were harvested and divided into two groups. The control group was planted and cultured in MEM plus 10 % FCS as described. The experimental group was cultured in MEM plus 10 % FCS treated by the addition of cortisone-21-phosphate to a final concentration of 50 μg/ml.

Twenty-four hours after planting, both groups were infected with accinia virus as described. After a 2.0 hour adsorption period, the acrophages were washed and fresh medium was added. The experimental roup medium received cortisone-21-phosphate at 50 μg/ml. Samples ere taken at various time intervals after infection and virus titra- tions were performed in MEF monolayers.

## RESULTS

### Morphology of Unstimulated Mouse Peritoneal Macrophages

Immediately after peritoneal lavage, mouse macrophages exhibited a spherical morphology with a prominent kidney-shaped nucleus. There was some size variation among the cells which was most noticeable right after planting. In general, the cell volume increased during cultivation and many older cultures usually showed a fair amount of uniformity in terms of cell size. Loss of cells from the surface of the coverslip during cultivation was commonly noted. This was most evident after several days *in vitro*.

Several hours after the initial culture many of the macrophages began to extend pseudopods and stretch out. Although a small number of cells retained their spherical morphology, the general trend during cultivation was observed as an increase in pseudopod extension.

Differing degrees of vacuolization was also noted among the cells. This phenomenon was usually most prominent in older cultures and differed in intensity from culture to culture.

### Vaccinia Infection of Mouse Peritoneal Macrophages

It has been shown by Ward (1980) that stimulated mouse peritoneal macrophages do not permit vaccinia replication *in vitro*. Because thioglycollate stimulated macrophages exhibit increased microbicidal activities (Mogensen, 1979), it was of interest to learn if unstimulated mouse macrophages could also prevent multiplication of IHD vaccinia.

Twenty-four hour cultures of mouse peritoneal macrophages were infected with vaccinia at a multiplicity of infection between 0.1 and 1.0. Growth assays titered in mouse embryo fibroblast monolayers

revealed a steady decline in viral titer during the 72 hour period (Fig. 1). Coverslips were stained for examination of the macrophage cultures at each of the sampling times. No demonstrable viral induced CPE was observed at any time during the infection period (Fig. 2-5).

Autoradiography

The fact that vaccinia virus replicates in the cytoplasm of susceptible cells makes it possible to determine the extent of viral DNA replication by autoradiography. When radio-labeled nucleotides are added to medium in coverslip cultures of vaccinia infected mouse macrophages, uptake of these nucleic acid precursors into the cytoplasm indicates vaccinia DNA replication and is detected by the induction of silver grain formation in overlayers of nuclear track emulsion.

Using this technique, Ward (1980) showed that no significant DNA synthesis occurred in vaccinia infected stimulated mouse peritoneal macrophages. Because of the similar inhibition of vaccinia multiplication by stimulated (Ward, 1980) and nonstimulated macrophages (Fig. 1), it was of interest to determine if vaccinia DNA synthesis is inhibited in unstimulated macrophages.

The results of autoradiography experiments indicated that no significant viral DNA replication occurred in cultured normal mouse macrophages (Fig. 6). Less than 1.0 % of the cells was observed to develop any cytoplasmic label.

Vaccinia Infection of Macrophages from Immature Mice

As noted previously, Johnson (1964) demonstrated a difference in the ability of macrophages from immature and adult mice to prevent HSV induced encephalitis. Experiments were undertaken to determine if a similar age-dependent phenomenon is demonstrable in this vaccinia-mouse macrophage model.

Figure 1. The inhibition of vaccinia virus by unstimulated mouse peritoneal macrophages.

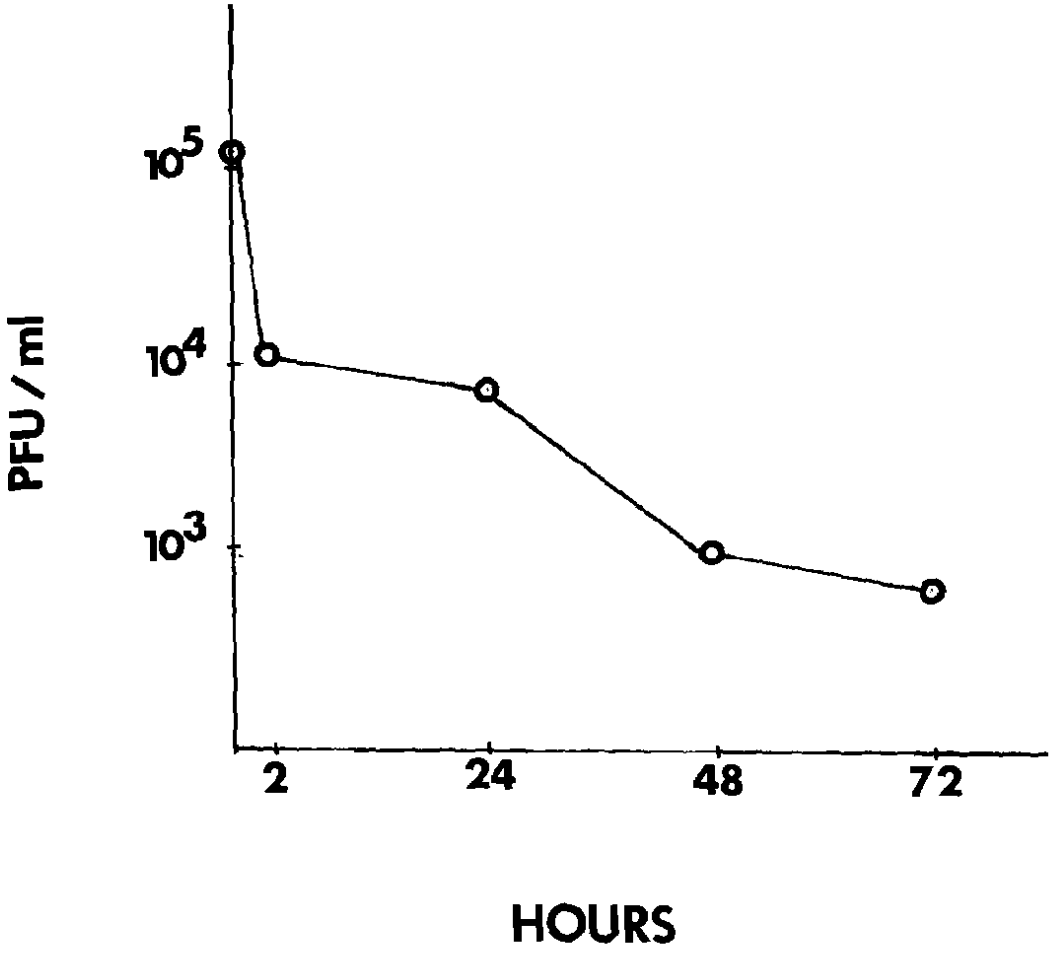
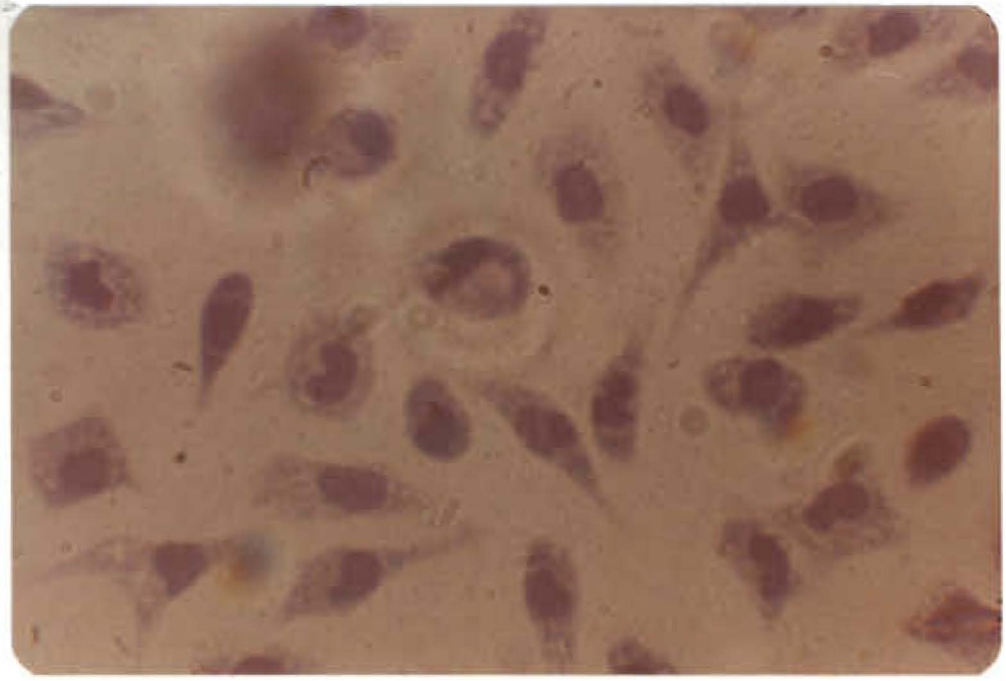

PFU / ml
$10^5$
$10^4$
$10^3$
2
24
48
72
HOURS

Figure 2. Adult mouse macrophages infected with vaccinia virus (0.1 MOI) at 14 hours post infection. (x 450) No viral induced CPE is exhibited.

Figure 3. Adult mouse macrophages infected with vaccinia virus (0.1 MOI) at 24 hours post infection. (x 450)



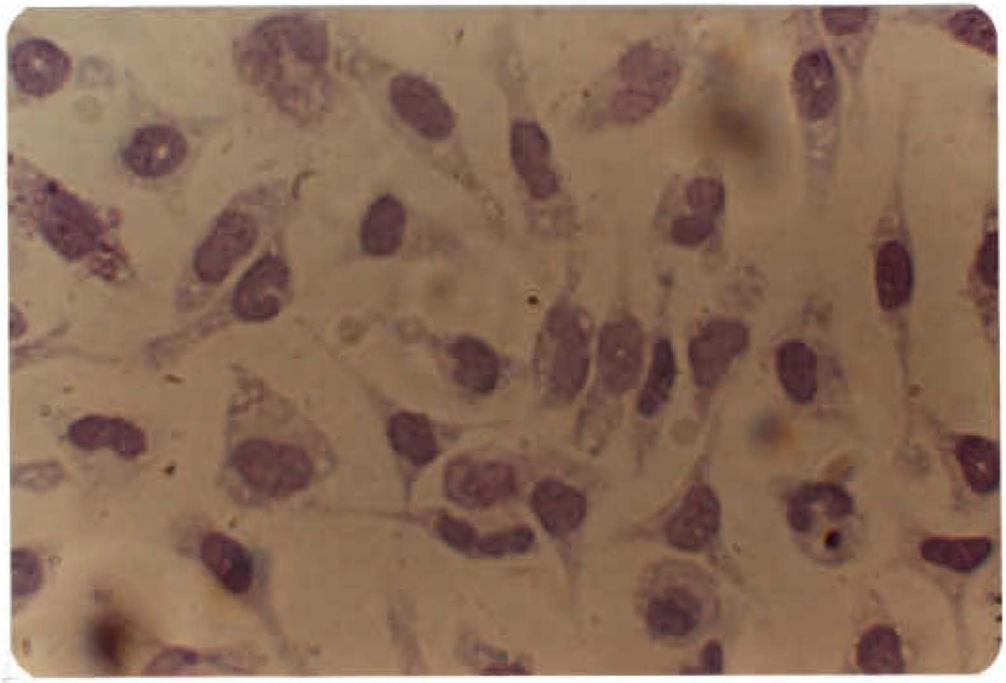
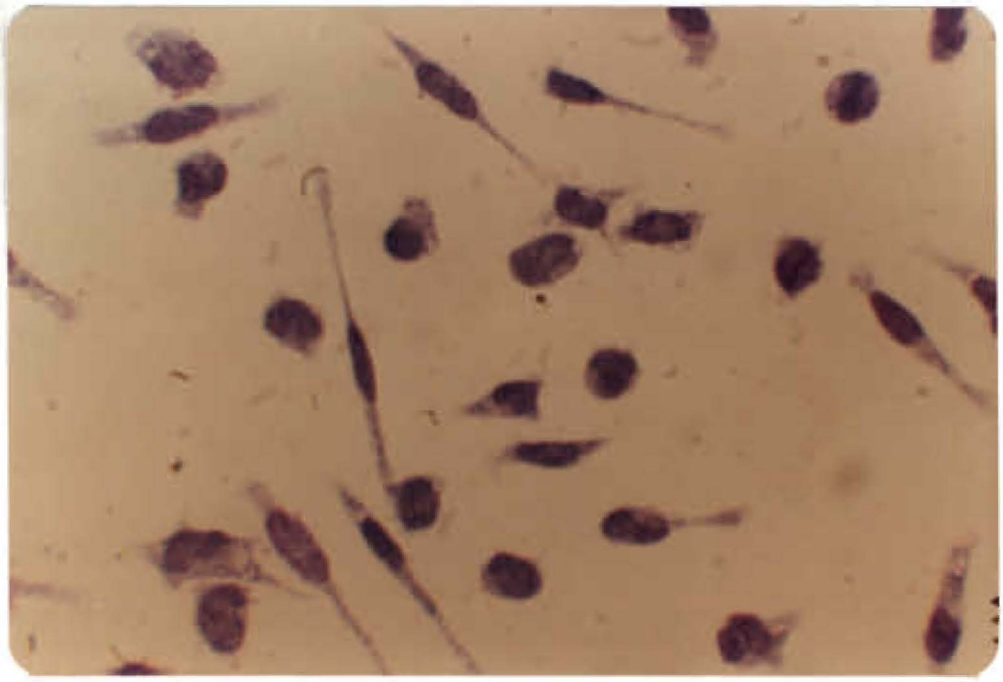


Figure 4. Vaccinia virus infected mouse peritoneal macrophages 48 hours post infection. (x 450) May Grünwald - Giemsa stain.

Figure 5. Infected mouse macrophages 72 hours after infection with vaccinia virus. (x 450) May Grunwald - Giemsa stain.



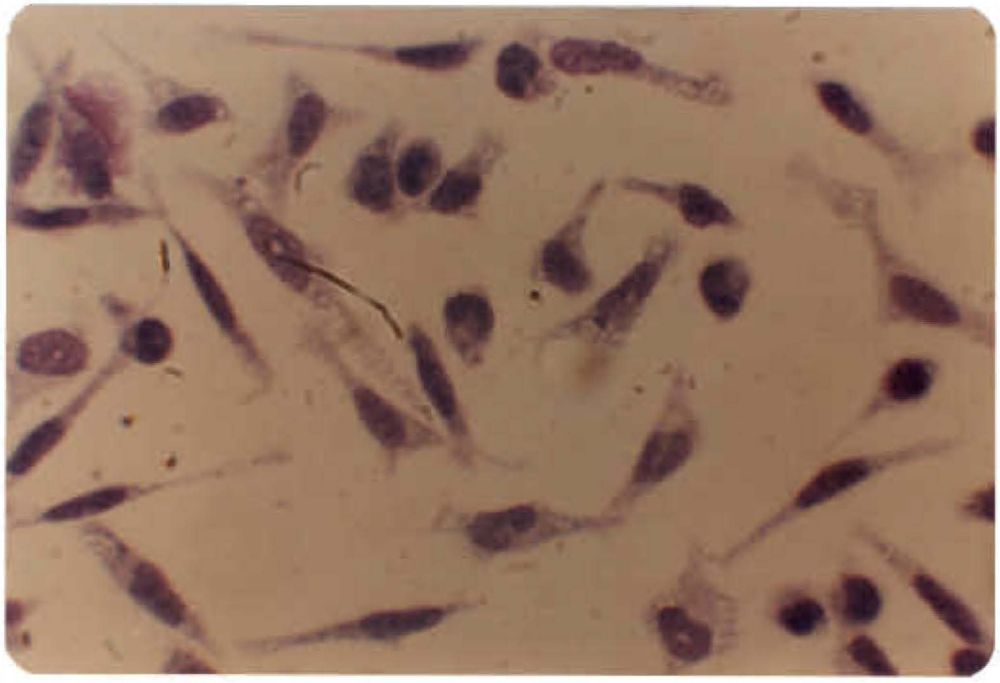
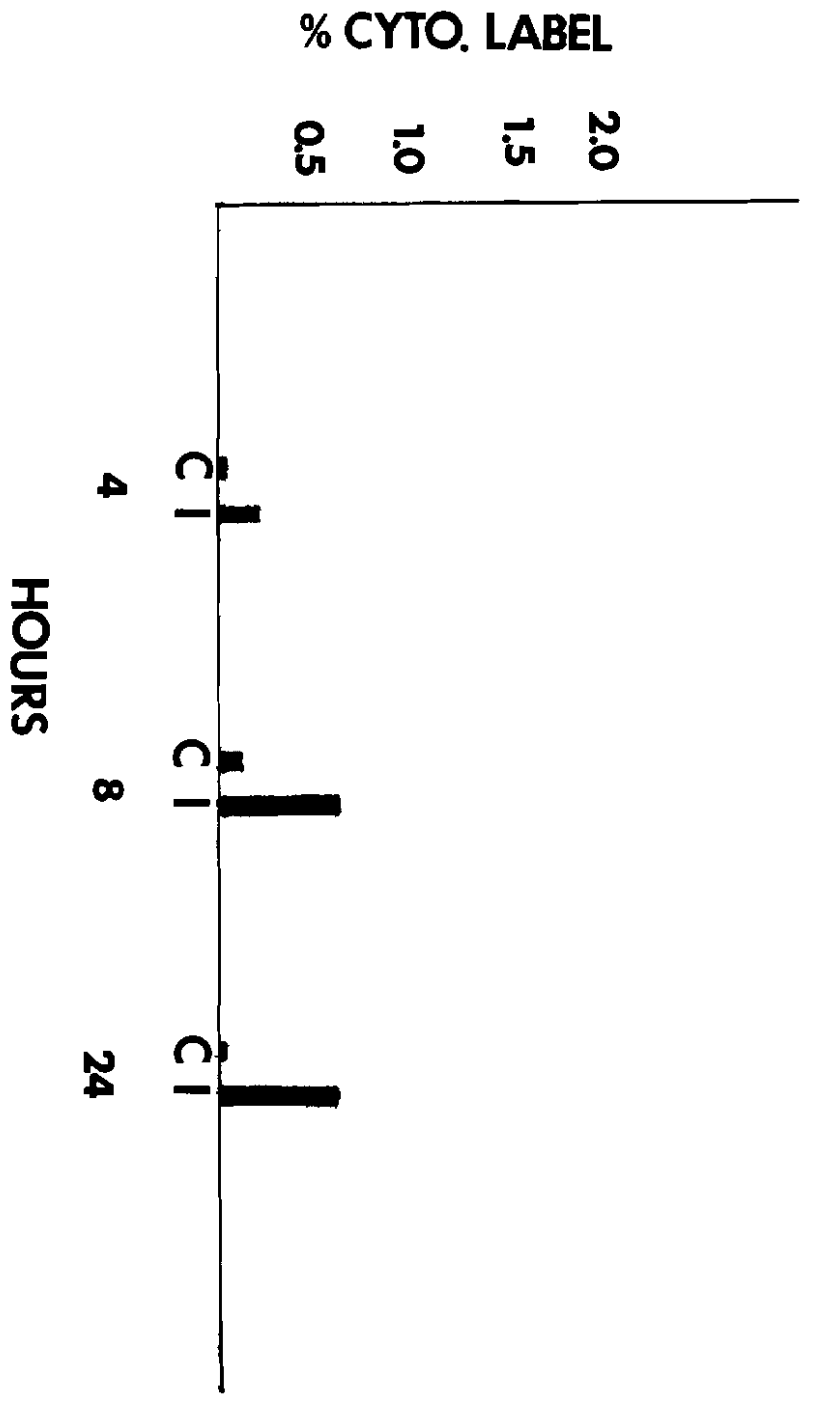


Figure 6. The ability of vaccinia virus to induce viral DNA synthesis in mouse macrophages expressed as the percentage of cells exhibiting cytoplasmic label.


% CYTO. LABEL
0.5
1.0
1.5
2.0
HOURS
4
8
24
C
I

Twenty-four hour cultures of macrophages from four week old mice were infected with IHD vaccinia virus at a multiplicity of infection of 0.1. A decrease in viral titer was noted as a function of time (Fig. 7). A great reduction in infectious virus was evident by 24 hours after infection. Thereafter the titer remained fairly constant for the duration of the experiment.

May Grünwald-Giemsa stained coverslips pulled at various sampling times revealed no viral induced CPE. The cells remained intact and active throughout the study period (Fig. 8-9).

Inactivation of Vaccinia Virus in Lysates of Rabbit and Mouse Macrophages

It has been shown that the IHD strain of vaccinia virus is capable of replicating in rabbit but not mouse peritoneal macrophages (Schultz, 1966). Because of this difference in susceptibility expressed by the macrophages, it was of interest to determine if macrophages from mice possess some extractable, viricidal component that rabbit macrophages lack.

Equal numbers of mouse and rabbit macrophages were subjected to four cycles of freeze-thaw to release intracellular components. Standard amounts of vaccinia virus were then added to the lysates and to a control tube containing only MEM. A gradual decrease in titer is seen in both systems during the first four hours of incubation followed by a sharper decline between four and six hours (Fig. 10) but no dramatic decrease in infectious virus was noted at any time during incubation.

Because there was no dramatic virus destruction in either lysate system, it was concluded that no highly viricidal molecule(s) are detectable by this procedure in macrophages from either species which

Figure 7. Inhibition of vaccinia virus replication by macrophages of four week old mice.

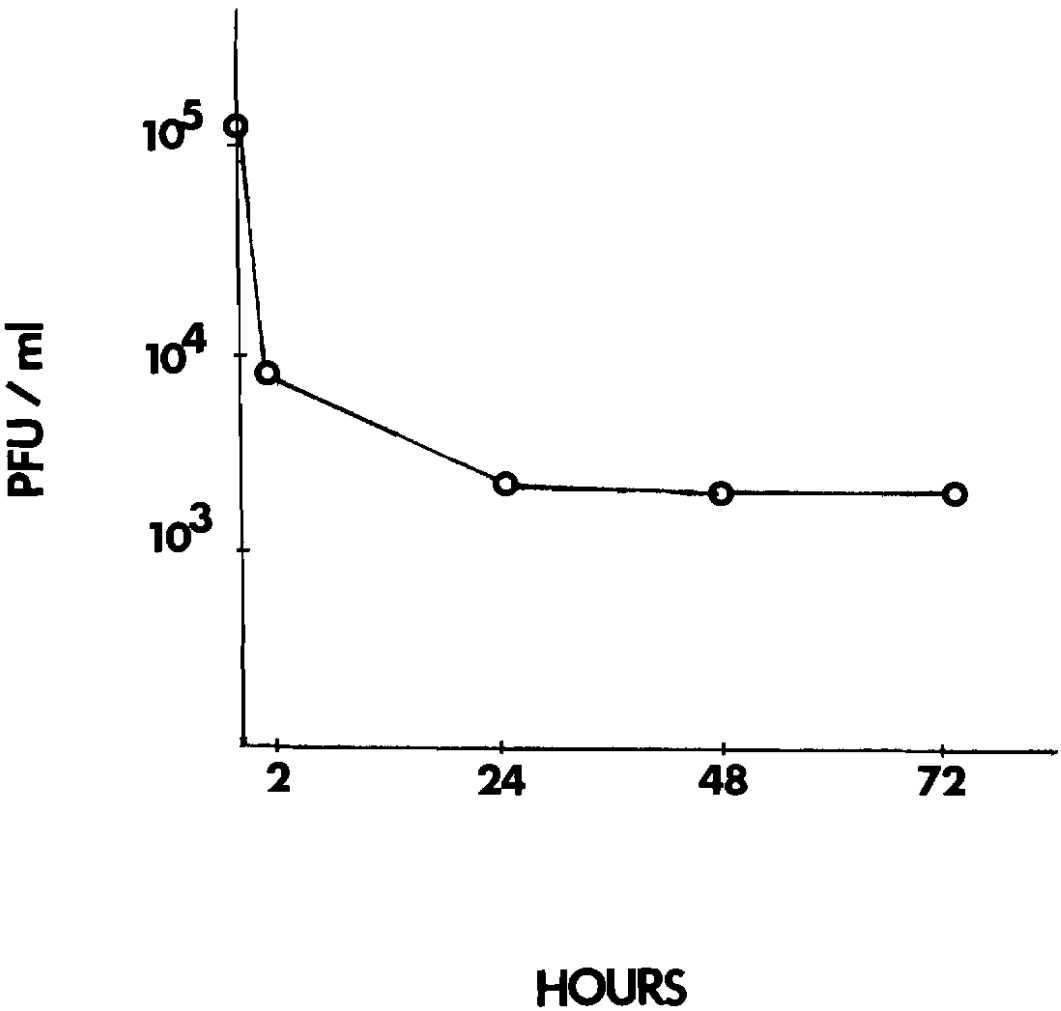
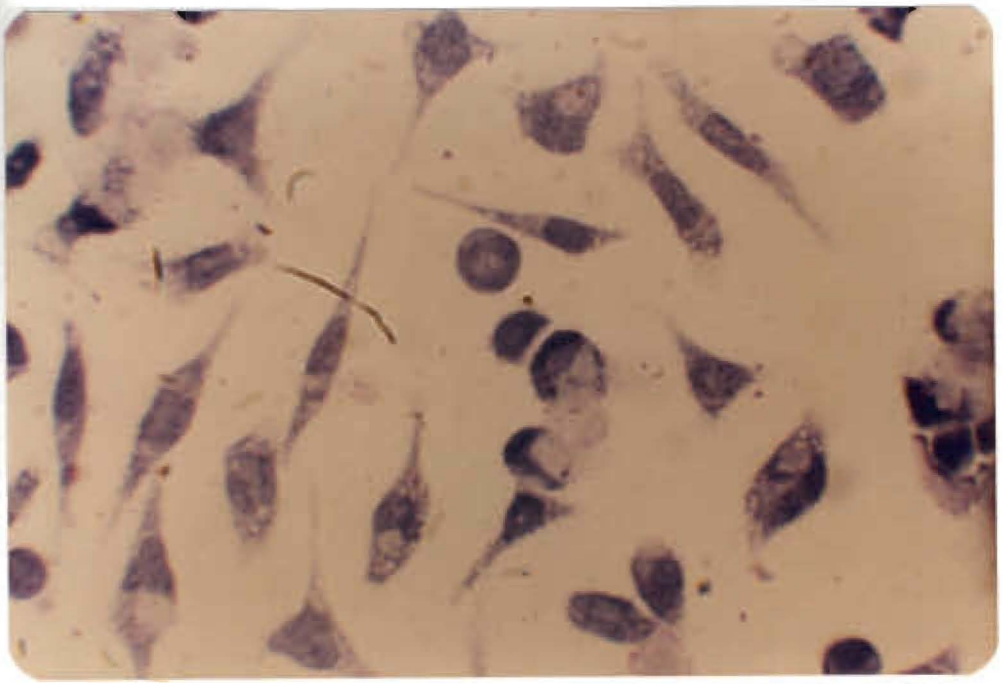

PFU / ml
$10^5$
$10^4$
$10^3$
2
24
48
72
HOURS

Figure 8. Uninfected macrophages from four week old mice after 24 hours in culture. (x 450) May Grünwald-Giemsa stain.

Figure 9. Macrophages from immature mice infected with vaccinia virus 96 hours post infection. (x 450) May Grünwald-Giemsa stain. No viral induced CPE is exhibited.



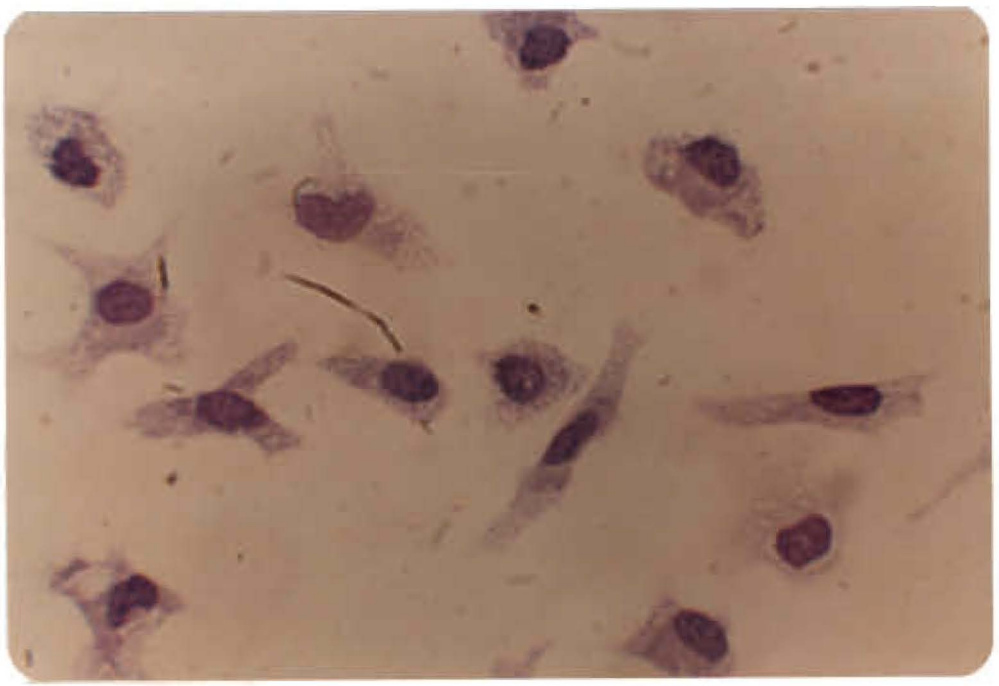
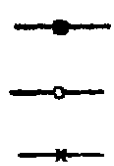
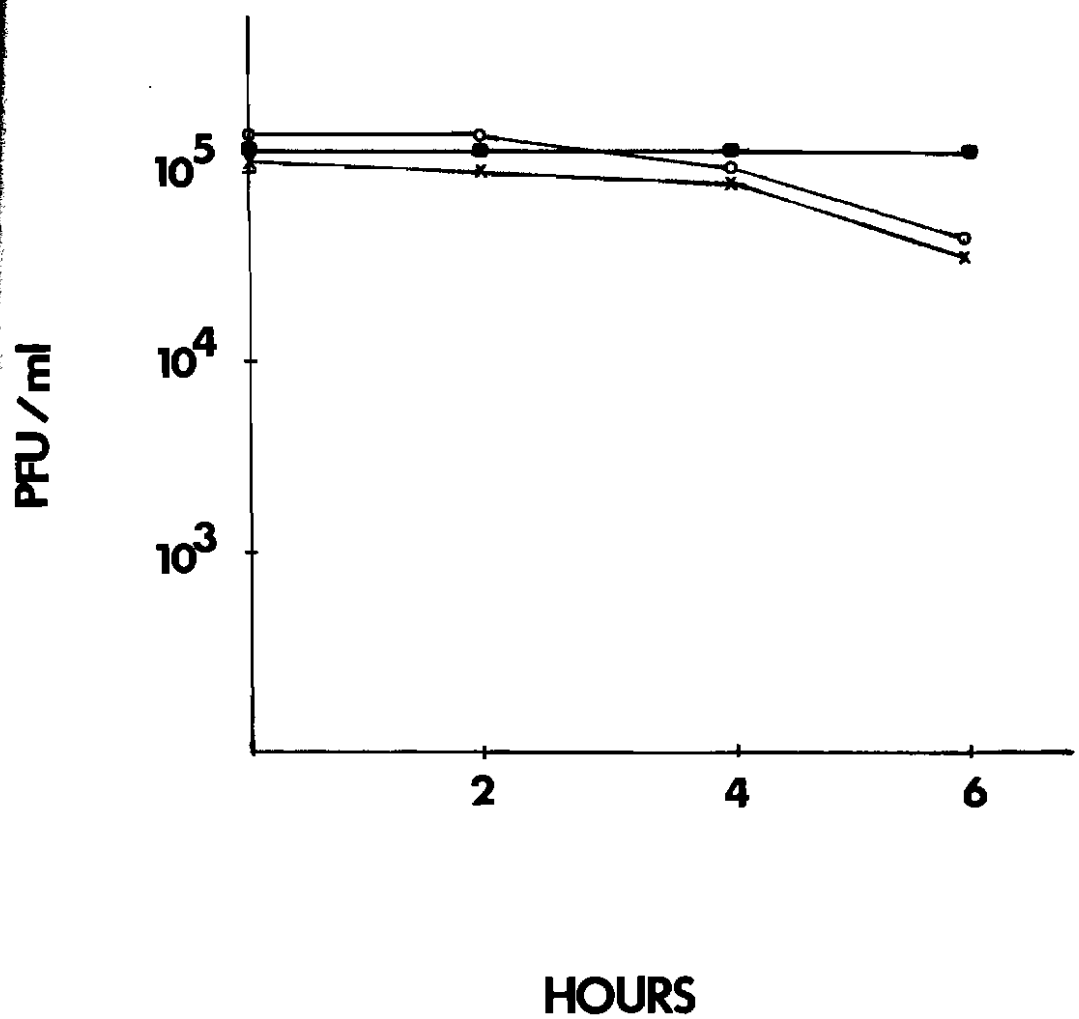


Figure 10. Ability of mouse and rabbit peritoneal macrophage lystates to inactivate vaccinia virus.




PFU/ml
$10^5$
$10^4$
$10^3$
2
4
6
HOURS

enable them to effectively inactivate vaccinia. Therefore, it was concluded the intact mouse macrophage must possess some mechanism or quality that enables it to inactivate IHD vaccinia.

Acid Phosphatase Staining of Peritoneal Macrophages

The activation of lysosomes in virus infected mouse macrophages was investigated as a possible antiviral mechanism. Macrophage lysosomal activation as expressed by increased lysosome membrane permeability was determined by a histochemical stain specific for acid phosphatase.

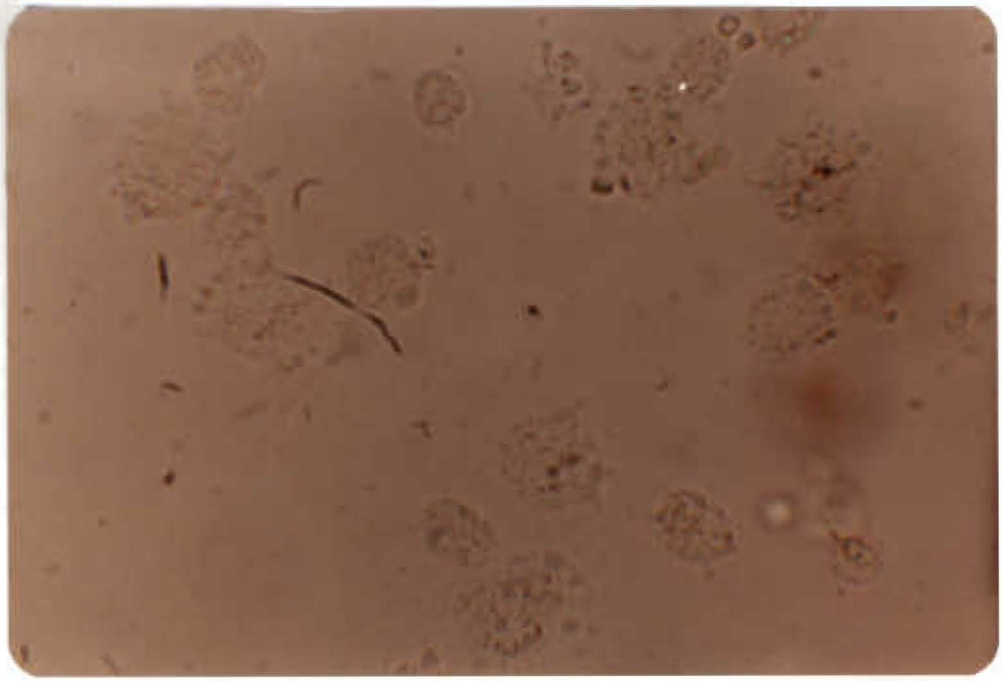
Coverslips from 24 hour cultures of mouse and rabbit macrophages were stained for acid phosphatase prior to infection. Control and infected macrophages were stained at 2.0, 4.0, and 8.0 hours after infection.

Rabbit peritoneal macrophages exhibited only a minimal amount of lysosomal activation in both control and infected systems and the amount of particulate staining did not increase with time (Fig. 11-14). However, mouse peritoneal macrophages exhibited a marked lysosomal activation as evidenced by intense granular staining in the cytoplasm. Lysosomes in virus infected mouse macrophages appeared to activate more quickly than those in uninfected controls. A darker, more intense staining reaction was noted in infected cells when compared to uninfected controls at 2.0 and 4.0 hours (Fig. 15-18). Larger granules, present in greater numbers in infected macrophages than in control slides, were interpreted as representing large vacuoles resulting from phagosome-lysosome fusion. However, very little difference was noted between the stain reaction in infected and control mouse cells by 8.0 hours post infection (Fig. 19, 20).

It was concluded from these studies that the ability of lysosomes

Figure 11. Rabbit peritoneal macrophages (uninfected) after two hours incubation exhibiting very little particulate staining. (x 450) Acid Phosphatase Stain.

Figure 12. Rabbit peritoneal macrophages two hours post infection with vaccinia virus. (450 x) Acid Phosphatase Stain.



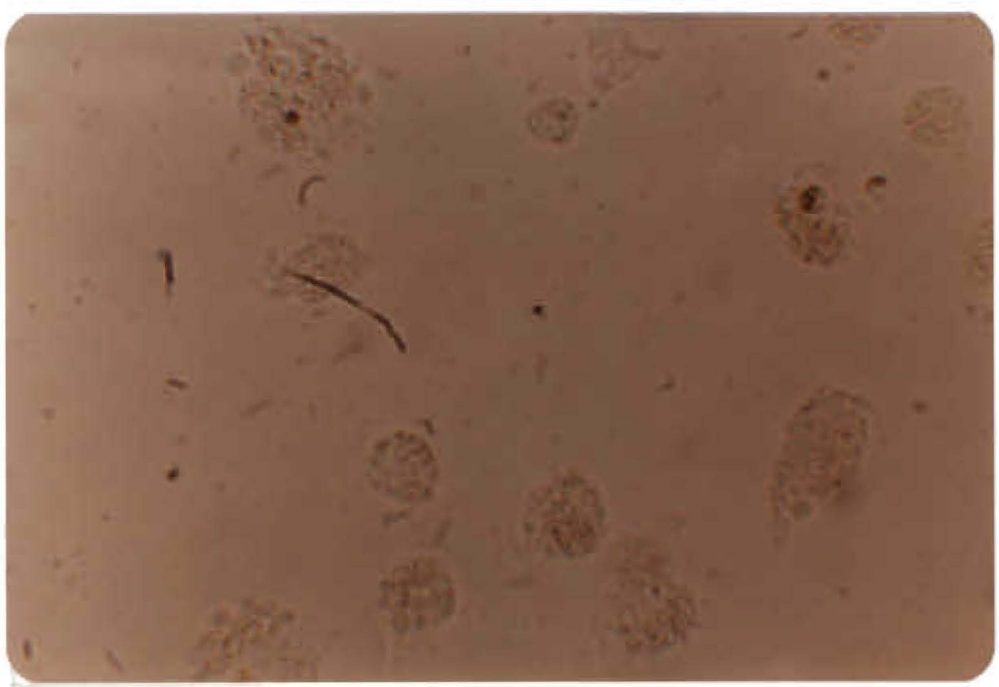
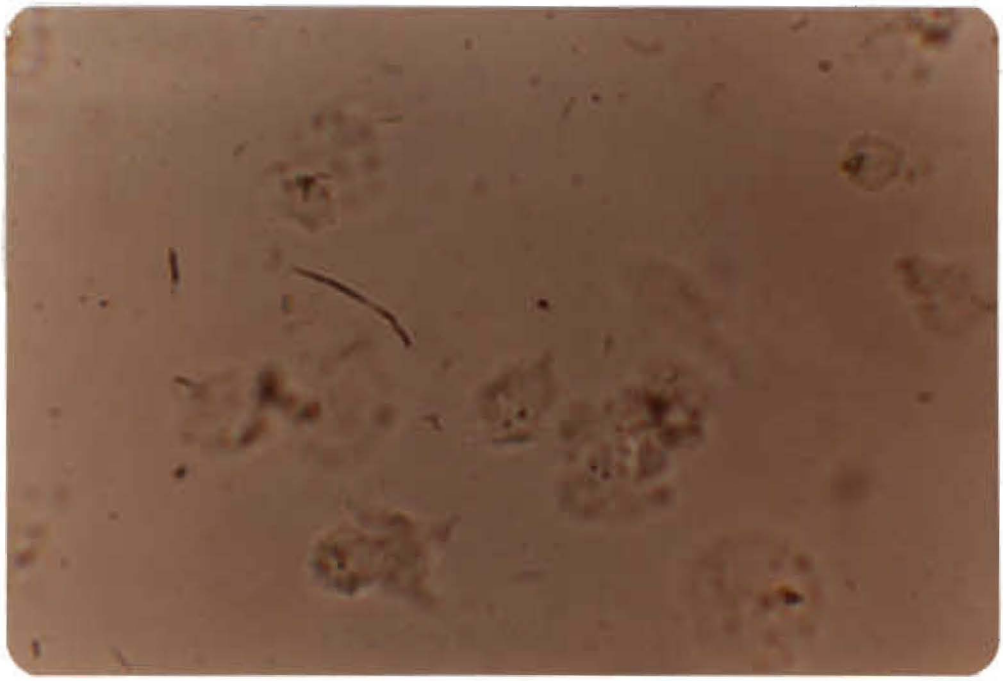


Figure 13. Uninfected rabbit peritoneal macrophages eight hours incubation (x 450) exhibiting very little particulate staining. Acid Phosphatase Stain.

Figure 14. Vaccinia infected rabbit peritoneal macrophages eight hours post infection. (x 450) Acid Phosphatase Stain.



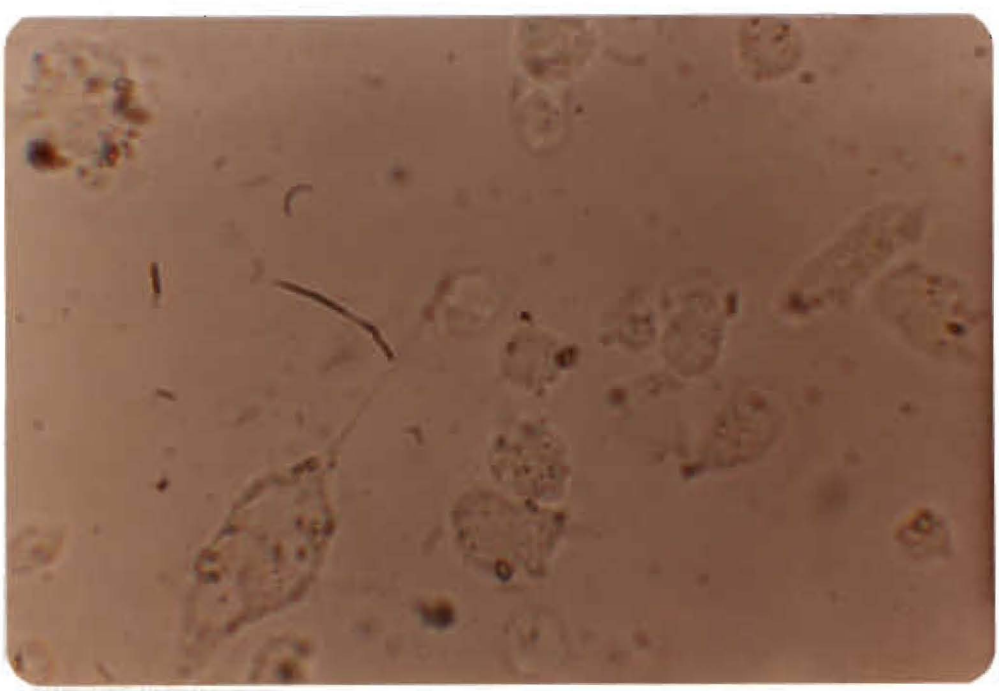
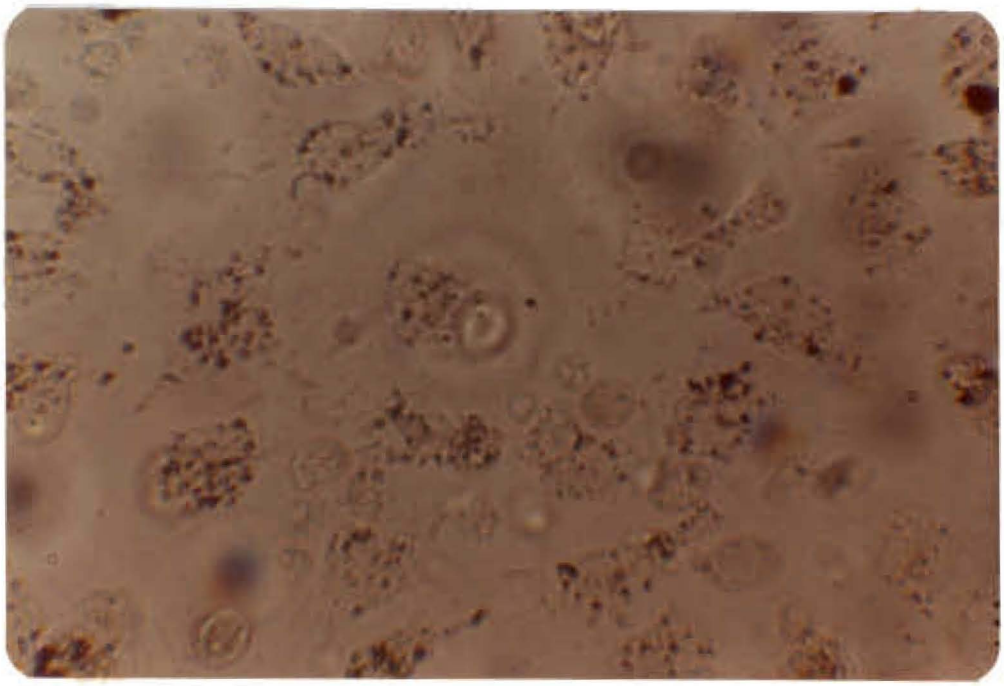


Figure 15. Uninfected mouse peritoneal macrophages after two hours incubation exhibiting slight particulate staining. (x 450) Acid Phosphatase Stain.

Figure 16. Vaccinia infected mouse peritoneal macrophages two hours post infection exhibiting marked particulate stain in cytoplasm. (x 450) Acid Phosphatase Stain.



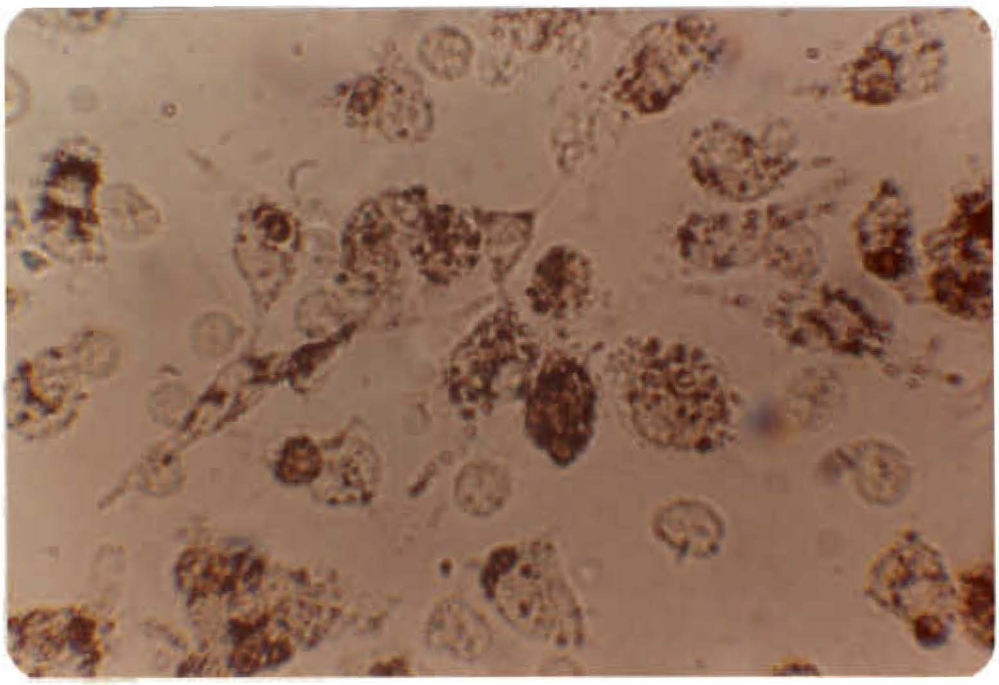
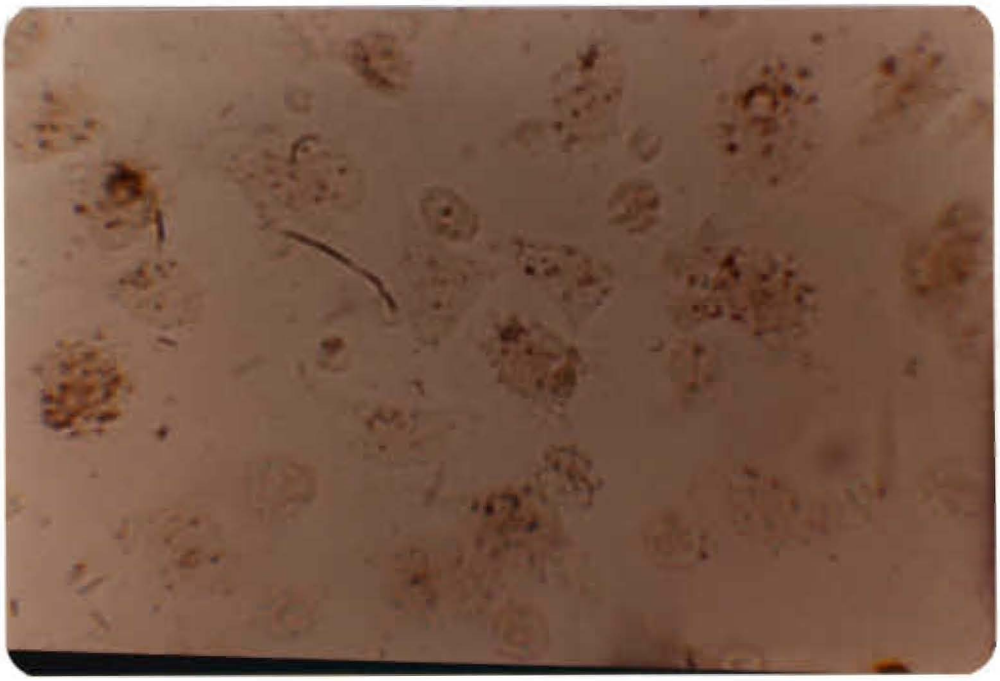


Figure 17. Uninfected mouse peritoneal macrophages after four hours incubation. (450 x) Acid Phosphatase Stain.

Figure 18. Infected mouse peritoneal macrophages exhibiting granular staining four hours post infection. (x 450) Acid Phosphatase Stain.



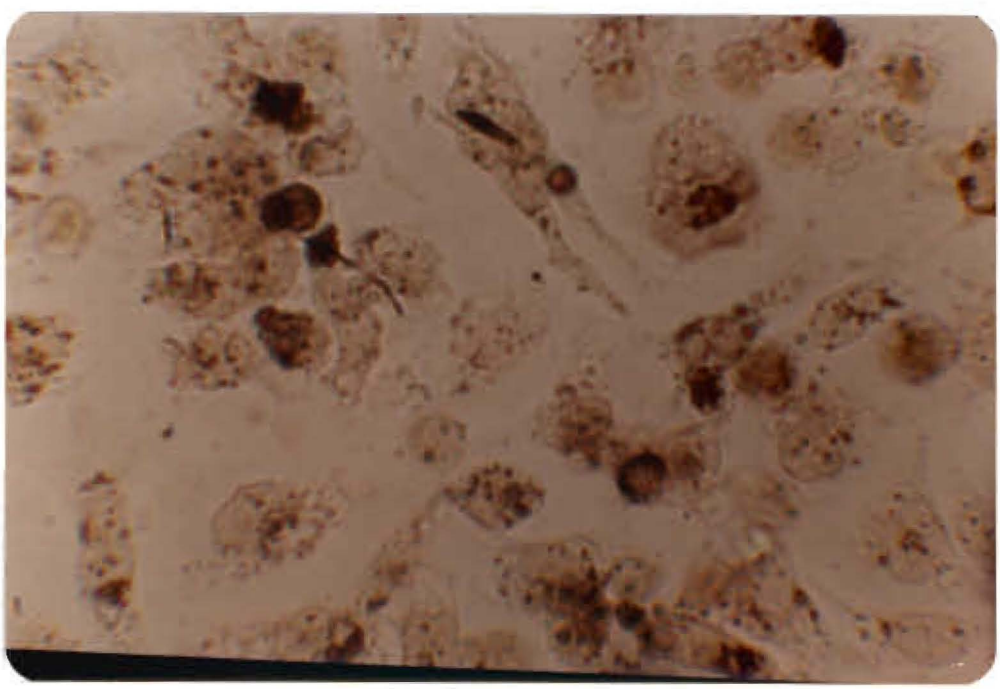
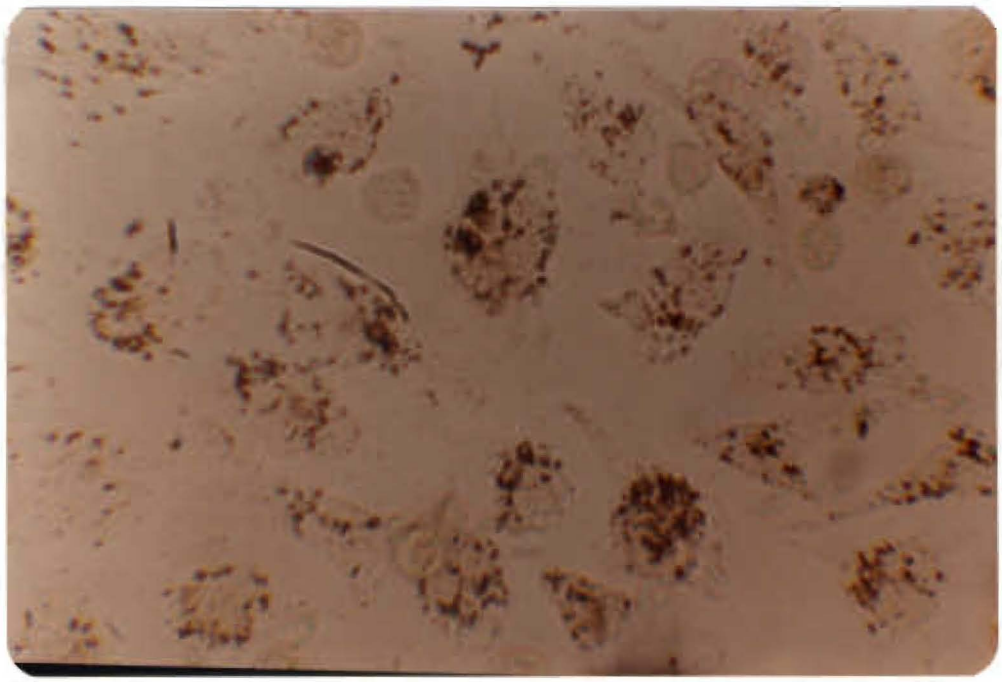
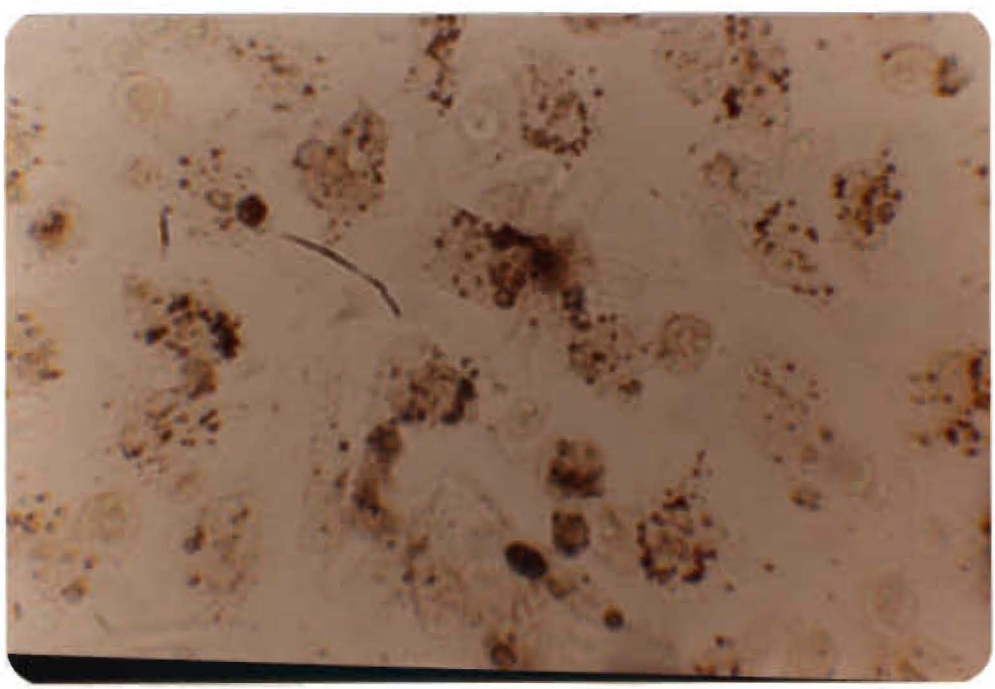


Figure 19. Uninfected mouse macrophages after eight hours incubation. (x 450) Acid Phosphatase Stain.

Figure 20. Vaccinia infected mouse peritoneal macrophages eight hours post infection. (x 450) Acid Phosphatase Stain.





from mouse macrophages to activate early in the infection may be a possible mechanism for their ability to resist vaccinia infection.

Cortisone-21-Phosphate Treatment of Mouse Peritoneal Macrophages

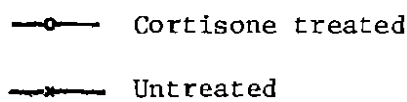
Cortisone has been shown to stabilize lysosomal membranes and interfere with lysosome-phagosome fusion (Merkow et al., 1968; Weissmann, 1969) in mouse and cat cells. Addition of cortisone-21-phosphate to MHV resistant macrophage cultures has been shown to render the phagocytes susceptible to virus infection (Gallily et al., 1964). An attempt was made to render mouse macrophages susceptible to vaccinia in a similar fashion by the addition of cortisone-21-phosphate *in vitro*.

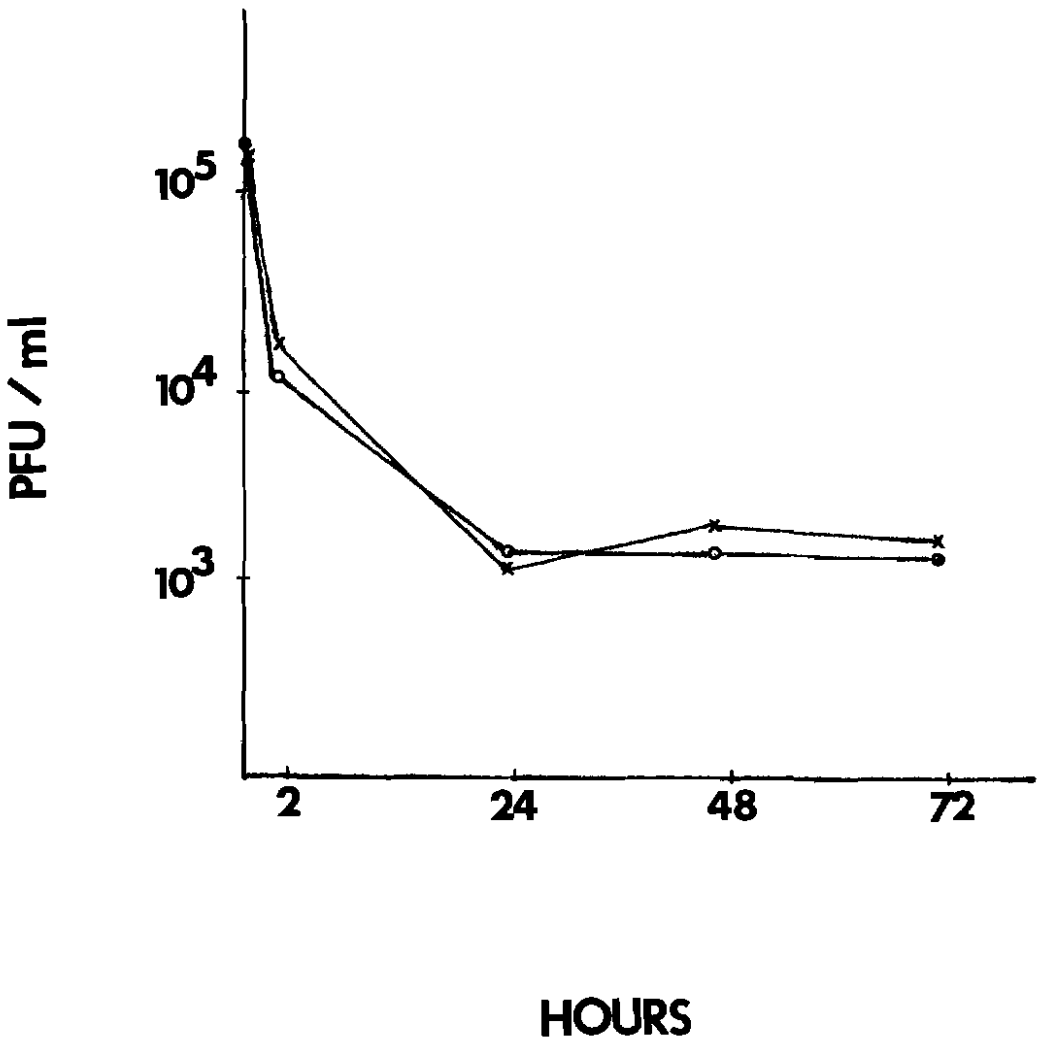
Cortisone-21-phosphate was added at a concentration of 50 μg/ml to freshly planted cultures of mouse macrophages. Twenty-four hours later, coverslips from untreated and cortisone treated cultures were pulled and stained for lysosomal activation with acid phosphatase stain. Tubes of macrophages from both systems were then infected with IHD vaccinia.

Acid phosphatase stained coverslips revealed a distinct particulate staining in both cortisone treated and untreated cultures. No differences in discrete particle formation or intensity of the reaction were observable in the two systems. This was interpreted as a failure of the cortisone-21-phosphate to stabilize the lysosomal membrane.

Viral titers from infected cultures pulled at various time intervals showed that vaccinia inhibition was similar in both cortisone treated and untreated macrophage cultures (Fig. 21). Coverslips stained with May Grünwald-Giesma revealed no viral induced CPE in either system at any time during the infection.

Figure 21. Inhibition of vaccinia virus replication by untreated and cortisone-21-phosphate treated mouse peritoneal macrophages.




PFU / ml
$10^5$
$10^4$
$10^3$
2
24
48
72
HOURS

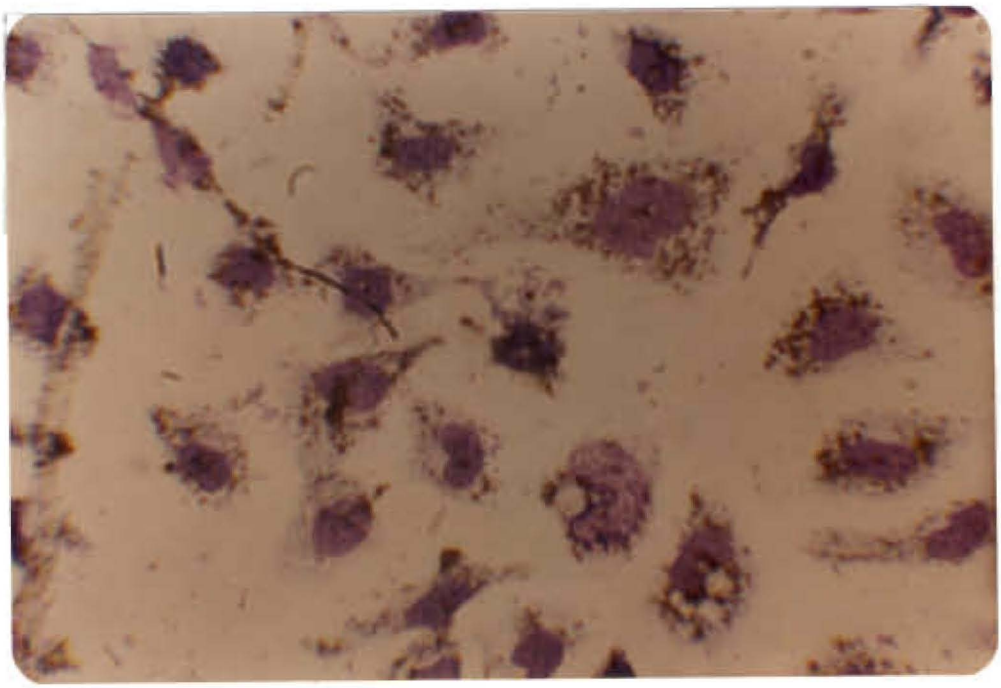
Carbon Uptake Studies

Vaccinia virus infections are initiated by the entry of virus into the cell by phagocytosis. Because this phagocytis event is so important to the infection process, it was necessary to determine if all macrophage populations used in this study exhibited high levels of phagocytosis. The phagocytic ability of these macrophage systems was determined by means of a carbon uptake assay.

Stained coverslips of cells from all systems showed numerous carbon particles in the macrophage cytoplasm. Also, the number of cells exhibiting carbon uptake in each system was very similar (Fig. 22-24). Four week, adult and cortisone treated macrophage cultures contained 97 %, 95 %, and 98 % phagocytic cells respectively. These data indicate that all cell systems contained a majority of highly phagocytic cells.

Figure 22. Four week old mouse macrophages showing ingestion of numerous carbon particles. (x 450) May Grünwald-Giemsa

Figure 23. Adult mouse peritoneal macrophages one hour after incubation with carbon particles. (x 450) May Grünwald-Giemsa Stain



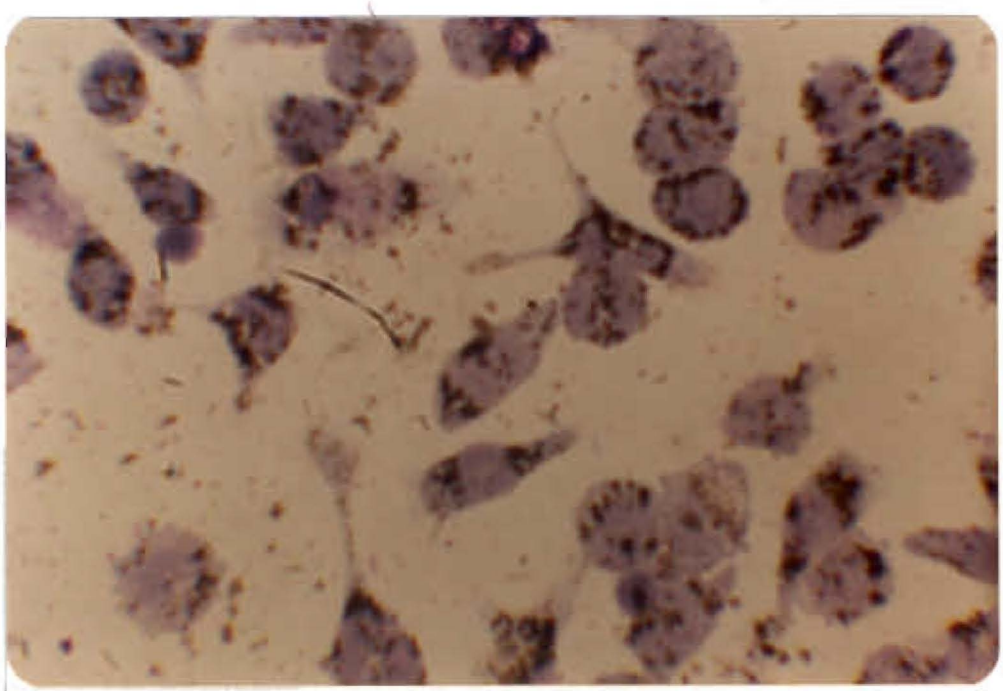
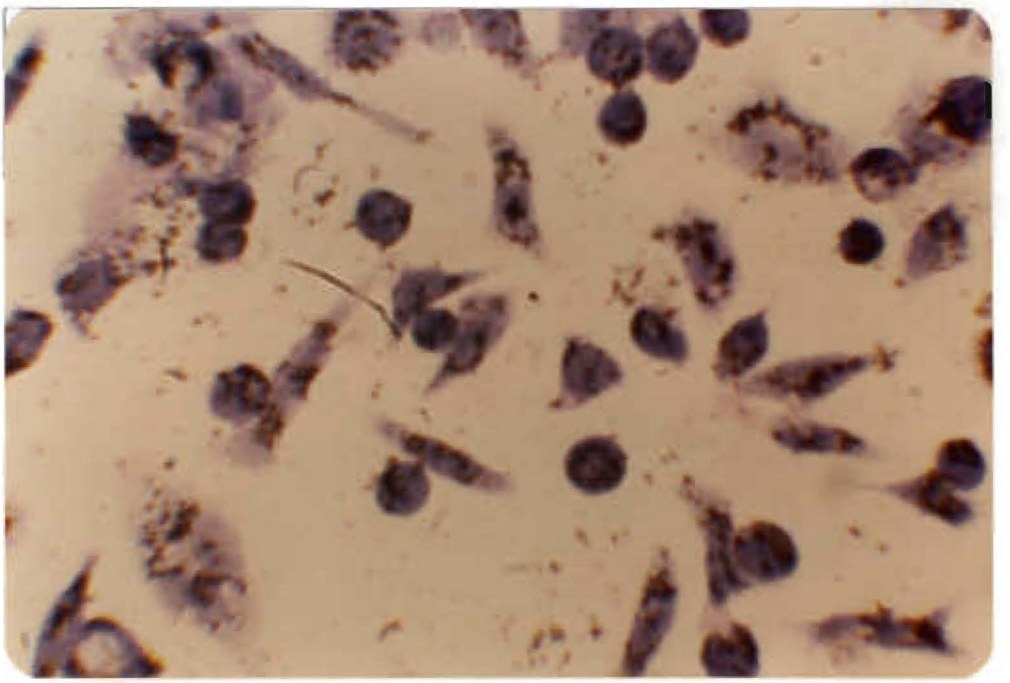


Figure 24. Mouse peritoneal macrophages treated with cortisone-21-phosphate showing phagocytosis of numerous carbon particles. (x 450) May Grünwald-Giesma



## DISCUSSION

Vaccinia virus is the type species for the pox virus group and as such has been characterized in terms of its structure, development, and antigenic structure. It is a large, complex, animal virus containing double stranded DNA. Vaccinia virus possesses a lipoprotein membrane surrounding a biconcave protein core. The core encloses the viral DNA. Two proteinaceous lateral bodies are found next to the biconcave surfaces of the core but the function of these two bodies is unclear (Briody, 1966; Silverstein, 1970).

The infection of cells with vaccinia begins with the adsorption of the virion to the cell membrane. Virus adsorption is a temperature and cellular energy independent event and does not appear to require the presence of specific receptors (Fenner et al., 1974).

Vaccinia penetrates the cell by phagocytosis and is observed to reside intracellularly in phagocytic vacuoles. The degradation of the viral envelope begins immediately after entry and is effected by cellular enzymes. First stage uncoating continues as the viral envelope interacts with the vacuolar membrane causing a disruption of both membranes and a release of the viral core into the cell cytoplasm. The mechanism involved in this interaction is not yet fully understood.

Viral messenger RNAs are released from the core into the cytoplasm where they initiate the production of virus specific enzymes by the host cell's synthetic equipment. Among the first enzymes produced is a protein which may be responsible for the second stage of viral uncoating. Following the release of the mRNAs, the viral core breaks down and releases viral DNA into the cytoplasm. At this time, host cell metabolism is directed towards the production of viral components. Virus assembly and maturation is accomplished in discrete areas in the

cytoplasm. Mature particles are released either by cell lysis or by direct passage into neighboring cells via intracellular bridges (Briody, 1966; Joklik, 1966; Penner et al., 1974).

Precise information concerning the replicative cycle of vaccinia virus has enabled investigators to determine the specific stage of infection at which viral inhibition occurs in immune cells. Buchmeier et al. (1979) used various uncoating assays, electron microscopy, and immunotechniques to study the inhibition of vaccinia multiplication by immune rabbit macrophages. It was determined that the macrophage, expressing acquired immunity, interfered in some way with a late step in viral assembly and the infection was aborted at this point.

Different mechanisms have been proposed for the inhibition of vaccinia replication by macrophages from genetically resistant mice. For example, Silverstein (1975) provided evidence that IHD vaccinia virus undergoes first stage uncoating and viral cores are released into the cytoplasm of mouse macrophages. Although some viral mRNAs were released into the cytoplasm, the mRNA coding for the uncoating enzymes was prevented in some manner from translating. It was suggested that second stage uncoating was prevented and the infection was aborted at this point.

Studies performed by Ward (1980) suggest a different scenario. Ward examined the cytoplasm of vaccinia infected mouse macrophages by electron microscopy for the presence of viral cores. Although extremely high multiplicities of infection were used, no viral cores were found in the cytoplasm. Moreover, the virus appeared to be degraded within the phagocytic vacuole.

The discrepancy between the two studies may be a result

of differences in the strain of mice or differences in the infectious dose utilized. It is clear that a good deal of disagreement exists concerning the mechanism of vaccinia inhibition utilized by mouse macrophages.

The interaction of unstimulated mouse macrophages with vaccinia virus was examined in this study. Normal mouse macrophage cultures were infected with non-toxic doses of IHD vaccinia virus to determine the ability of the virus to replicate in this cell type. It is evident (Fig. 1) that virus titers fall significantly during the 72 hour infection period and that vaccinia fails to replicate in this system. These results are in agreement with those obtained by other investigators (Nishmi and Niesikowski, 1963; Ward, 1980). The continuing presence of infectious virus in macrophage cultures at 72 hours may represent cell membrane bound or unadsorbed virus in the medium, or virus produced by non-resistant cells contaminating the culture.

Autoradiography studies were utilized to determine the extent, if any, of viral DNA replication in unstimulated mouse macrophages <u>in vitro</u>. Examination of the prepared slides revealed no significant label of DNA replication (Fig. 2). No more than 0.6 % of the macrophages observed contained demonstrable cytoplasmic labeling. This finding is in accord with results of similar studies by Ward (1980).

Macrophages obtained by the method of peritoneal lavage contain a variety of cell types. Although the majority of the cells are macrophages, other cells such as lymphocytes, fibroblasts, blood leukocytes, mast cells, and adipose cells contaminate the cultures.

The small numbers of cells which allowed viral DNA replication may represent non-resistant cells, such as fibroblasts, which contaminated the culture. However, in most cases it was impossible to

discern labeled cells from surrounding macrophages on the basis of morphology. Thus, they may represent a subpopulation of macrophages, or morphologically similar cells, which are susceptible to vaccinia infection. It must be emphasized that the overwhelming number of cells did not allow viral DNA replication. It is concluded that the inhibition of replication of vaccinia in mouse macrophages occurs at some point before viral DNA replication.

Studies dealing with age-dependent resistance have revealed deficiencies in the ability of macrophages from immature mice to resist virus infection. Mogensen (1978) studied HSV-2 virus infections in immature and adult murine macrophages removed from mice genetically resistant to HSV-2 induced hepatitis. His results indicate that macrophages from newborn mice did not restrict HSV-2 as compared to macrophages from adult mice. Studies by Gallily (1967) indicate that $C_3H$ mice which are genetically resistant to MHV-2 are not fully resistant to this virus until the age of 14 days. This age dependent development of resistance is mirrored by the ability of macrophages to inhibit viral growth. Thus the mechanism responsible for the genetic resistance of this strain is still developing during the first few weeks of life. Because these and other studies (Hirsch, et al., 1970; Johnson, 1964) have indicated an increased susceptibility of macrophages from immature mice to viruses, it was of interest to determine if a similar phenomenom was demonstrable with vaccinia virus.

Mouse macrophages from four week old individuals were observed to inhibit vaccinia multiplication during a 96 hour infection period (Fig. 3). Observations of stained coverslips from 96 hour infected cells seem to show no viral induced CPE or toxic effect. These results seem

to indicate that the natural resistance of mouse macrophages to vaccinia virus is expressed prior to the fourth week of life. Clearly more studies are needed to establish the exact age at which the development of this natural resistance develops and what maturational processes are required before it is expressed.

Peritoneal macrophages possess an impressive array of lysosomal acid hydrolases. There are more than 40 acid hydrolases in lysosomes including such enzymes as phosphatases, aryl sulfatases, phospholipases, and proteinases (Werb, 1982). The discharge of these hydrolytic enzymes into particle containing phagosomes and the rapid acidification of the vacuole have been implicated as possible antimicrobial mechanisms (Nathan et al., 1980; Werb, 1982).

Indirect evidence for the importance of these enzymes in bacterial infections is given by studies showing increases in lysosomal enzyme content in macrophages arriving at the site of bacterial implantation (Mackaness and Raffel, 1971). The involvement of hydrolytic enzymes in host defense against virus infection has been suggested by some studies. For example, investigations by Metha and Webb (1982) have demonstrated the importance of lysosomal enzymes in Semliki forest virus infections of mouse macrophages. It was found that normally avirulent Semliki forest virus could infect and multiply in soluble gold blockaded macrophages. A close correlation between rising virus titers and suppression of lysosomal enzymes was noted. It was suggested that early digestion of virus by blockaded macrophages was inadequate and resulted in the multiplication of virus.

The studies of Schultz (1966) and Ward (1980) have implicated the involvement of lysosomal hydrolases in mouse macrophage inhibition of vaccinia infection. It was of interest to study this aspect of the

virus-macrophage interaction to determine its role as a possible mechanism of antiviral activity.

Experiments were performed in order to determine if viral growth inhibition in mouse macrophages may be due to some extremely efficient viricidal cellular molecule(s) which are capable of inactivating vaccinia. Cell lysates were prepared from equal numbers of resistant mouse macrophages and susceptible rabbit macrophages. No dramatic drop in virus titer was observed in either mouse or rabbit macrophage lysates. The results of this experiment indicate that virus growth inhibition in mouse macrophages is probably not due to one or more soluble viricidal cell components. It appears that mechanisms expressed in the intact cell render the cell either susceptible or resistant to infection by vaccinia virus. Such processes would ultimately fall under the control of the animal's genome.

Lysosomal activation was studied histochemically in both mouse and rabbit macrophages by means of an acid phosphatase stain. A slight particulate staining was observed in both systems prior to addition of virus. Lysosomes in infected mouse macrophages appeared to activate quickly as evidenced by intense particulate staining at 2.0 and 4.0 hours. Control converslips were also observed to activate, but the reaction appeared to require a longer period of time for completion. Very little difference was noted between the staining reaction in infected and control mouse cells by 8.0 hours post infection. Infected and control rabbit macrophage lysosomes failed to activate to any significant degree during the time course of the experiment. Little particulate staining was observed at any sample time.

Studies by Cohn and Benson (1965) have indicated that pinocytosis

of calf serum stimulates lysosomal enzyme synthesis in macrophages cultured in high concentrations of calf serum. Similar studies by Axline and Cohn (1970) have indicated that the phagocytosis of digestible substrates also results in increased lysosomal enzyme production by macrophages. This phenomenon appears to be influenced by the quantity and digestibility of the substrate. It could be that the endocytosis event also stimulates lysosomal activation in mouse macrophages. This may explain the activation of control mouse lysosomes by 8.0 hours. MEM plus 2.0 % fetal calf serum (FCS) was used as the infection medium in these experiments. Activation of infected mouse lysosomes accelerates at this time probably because of the ingestion of vaccinia virions. Two hours later, this medium was replaced with fresh MEM containing 10 % FCS. This increased serum concentration at 2.0 hours may result in an activation of control lysosomes, similar to the activation of infected macrophage lysosomes.

These results suggest that the ingestion of vaccinia virus by mouse macrophages causes an early, increased level of lysosomal activity above that of uninfected controls. It is also evident that the lysosomes of mouse macrophages are more easily activated than those of rabbit macrophages. This mechanism expressed in mouse macrophages may subject phagocytized vaccinia to volley after volley of degradative enzymes and acid pH which render it noninfectious before first stage uncoating can occur. This would explain the absence of viral cores in the cytoplasm as described by Ward (1980). The lack of such a mechanism in normal rabbit macrophages may explain their susceptibility to vaccinia infection. If lysosomes are not activated by the phagocytosis of vaccinia particles then lysosome-phagosome fusion may occur so infrequently that the virus is not exposed to great amounts of hydrolytic

enzymes. If the virus is not destroyed at this point then first stage uncoating may ovvur and an infection may result.

Corticosteroids are known to have adverse effects on the host's ability to deal with viral infections. For example, Kass and Finland (1958) reported a complication of vaccinia and varicella infections in human patients undergoing corticosteroid therapy. These compounds have also been shown to depress the innate resistance of mice to various viruses. Examples of this phenomenon include studies in which coxsackie virus resistant mice were made susceptible by treatment with corticosteroids (Kilbourn and Horsfall, 1951). In addition, peritoneal and liver macrophage cultures from mice genetically resistant to mouse hepatitis virus (MHV) have been converted to susceptible cells by the addition of cortisone-21-phosphate (Gallily et al., 1964). These compounds are also known to stabilize lysosomal membranes (Weissman, 1969) and inhibit phagosome-lysosome fusion in murine alveolar macrophages (Merkow et al., 1968). Because of these phenomena, it was of interest to determine if corticosteroid treatment of mouse macrophages may render them susceptible to vaccinia virus. It was of particular interest to determine if treatment with cortisone-21-phosphate could interfere with lysosomal activation and thus alter the innate resistance.

Acid phosphatase stains of cortisone-21-phosphate treated and untreated macrophages revealed no significant difference in lysosomal activation. Furthermore, viral growth studies reveal a similar decrease in viral titer in cortisone treated and untreated macrophages. Therefore, it was concluded that cortisone-21-phosphate had no effect on the outcome of vaccinia infection of mouse macrophages.

It is important to note that the concentration of cortisone-21-phosphate used in this study (50 μg/ml) is greater than that utilized

by Gallily et al. (1964) (0.25 µg - 10.0 µg/ml). Cortisone at this greater concentration did not render mouse macrophages susceptible to vaccinia. The conflict between these results and those of Gallily is probably due to differences in virus and mouse strain studied. It may also indicate that the mechanism by which macrophages inhibit virus multiplication differs according to the virus involved.

Although cortisone treatment failed to render mouse macrophages susceptible to vaccinia virus infection, this does not disprove the suggestion that enhanced lysosomal activation may be the mechanism responsible for vaccinia inactivation by mouse macrophages. Lysosomal activation was observed in the cortisone treated cells and could have been responsible for the resistance in the treated cells. Lysosomal activation in cortisone treated cells may indicate a resistance in this particular group of mice to the membrane stabilizing effects of cortisone. Clearly, other corticosteroids should be tested for their ability to stabilize mouse macrophage lysosomal membranes.

Although the results of this study suggest that the level of lysosome activation may be the mechanism by which mouse macrophages inhibit vaccinia replication, other mechanisms may be involved and cannot be discounted. Interferon production by peritoneal macrophages has been reported (Fenner et al, 1974). It has been suggested that the production and release of interferon by macrophages early in the infection may play a role in resistance to virus infections. It is conceivable that this type of phenomenon may be involved in the resistance of mouse macrophages to vaccinia virus. If the first few macrophages to phagocytize vaccinia produced interferon, the remainder of the cells may be protected by the subsequent uptake of this compound. However, conflicting reports of interferon production by vaccinia infected mouse

macrophages have been published. Glasgow (1966) reported that peritoneal cells from vaccinia immune mice produced interferon, whereas Subrahmanyan and Mims (1970) could demonstrate no interferon production in similar studies. It is evident that there is not total agreement on interferon production in vaccinia infected mouse macrophages.

Macrophages have also been shown to undergo a respiratory burst associated with ingestion of particles similar to that exhibited by polymorphonuclear leukocytes. Activation of the hexose monophosphate shunt, increased oxygen consumption, and the generation of super oxide anion, hydrogen peroxidase, hydroxyl radical, singlet oxygen, and other metabolites of oxygen have been associated with this respiratory burst (Werb, 1982). These metabolites have been shown to have antimicrobial and antitumor activities. Although the antimicrobial role of these agents has been implicated in infections of mouse macrophages with microbes such as the protozoa Toxaplasma gondii (Nathal et al., 1980), their role in viral infections has not been well studied. Further studies concerning the ability of these compounds to act as viricidal agents or augment lysosomal enzyme killing of vaccinia virus is needed.

Dales (1965) suggested the interesting possibility that some component of the vaccinia envelope may be required for the membrane interaction and core release into the cytoplasm during first stage uncoating. Evidence for this is provided by the fact that heat denatured or antibody neutralized vaccinia virus is taken up and degraded in the phagocytic vacuoles of rabbit macrophages. Obviously, these alterations of the virus envelope have allowed a normally susceptible cell to inactivate vaccinia. If such a component exists, it could also help to explain the differences in resistance of mouse and rabbit macrophages to vaccinia virus. It could be that such a component would be incapable of reacting

with mouse vacuole membranes. In this case first stage uncoating would not occur and hydrolytic enzymes would inactivate the virus. Similarly it could be that the mouse lysosomal activation with a subsequent discharge of enzymes into the phagosome destroys this component and thus prevents first stage uncoating.

Dales' theory does have some problems associated with it. First, no such receptor has been demonstrated. Also, the phagocytic membrane is essentially the cellular plasma membrane folded around the virus. If such a receptor exists, first stage uncoating could occur extracellularly. Electron microscopy techniques have never demonstrated such an event.

The results of this study suggest that the ability of mouse macrophages to inhibit vaccinia virus may be due to the mechanism of lysosomal activation. Furthermore, this genetic resistance against IHD vaccinia is expressed in macrophages from four week old mice, indicating an early development of resistance. Further study concerning the role of interferon and oxygen metabolites in this resistance should be pursued.

## SUMMARY

1. Results from virus growth assays indicate that vaccinia virus is incapable of multiplying in unstimulated mouse macrophages.
2. Autoradiography studies of infected mouse macrophages show that no significant viral DNA synthesis occurs. This indicates that the block of virus replicative cycle occurs very early during the infection.
3. Viral growth assays with macrophages from immature mice indicate that the innate resistance against vaccinia virus is present by the fourth week of life.
4. Incubation of vaccinia virus in susceptible rabbit macrophage or resistant mouse macrophage lysates seem to show that the intact cell is responsible for the expression of resistance or susceptibility to vaccinia infection.
5. Acid phosphatase staining techniques revealed the activation of lysosomes in vaccinia infected mouse but not rabbit macrophages. This suggests a possible role of lysosome activation in the natural resistance of mice to vaccinia virus.

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