

AN ABSTRACT OF THE THESIS OF

Fatah Kashanchi for the Master of Science

in Biology presented on 17 May 1986

Title: A Comparative Study of Early Events In Resistant  
And Susceptible Macrophages Infected With Vaccinia Virus

Abstract approved: *Aileen McElree*

An investigation of the mechanism of resistance expressed by normal mouse macrophages against IHD vaccinia virus infection was conducted. Results from virus growth assays showed that vaccinia virus is incapable of multiplying in unstimulated mouse macrophages and instead is degraded by them. Studies concerning first stage uncoating of virus using sodium suramin as a host lysosome stabilizer indicated no change in viral titers and acid phosphatase particulate staining. This indicated that first stage uncoating of virus was not achieved due to lack of phagosome-lysosome fusion in the presence of suramin.

Electron micrograph studies revealed the presence of cytoplasmic cores, indicative of first stage uncoating, in both resistant and susceptible cells. Further studies concerning the second stage of uncoating indicated presence of degraded radioactive viral nucleotides in the supernatant of both mouse and rabbit peritoneal macrophages.

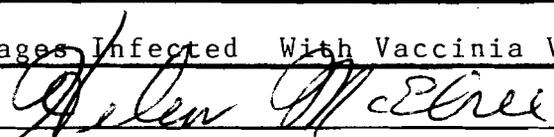
Autoradiography studies of infected mouse and rabbit macrophages showed that no significant viral DNA synthesis occurred in mouse cells when compared to rabbit cells. This indicated that the block of the virus replicative cycle in

AN ABSTRACT OF THE THESIS OF

Fatah Kashanchi for the Master of Science

in Biology presented on 17 May 1986

Title: A Comparative Study of Early Events In Resistant  
And Susceptible Macrophages Infected With Vaccinia Virus

Abstract approved: 

An investigation of the mechanism of resistance expressed by normal mouse macrophages against IHD vaccinia virus infection was conducted. Results from virus growth assays showed that vaccinia virus is incapable of multiplying in unstimulated mouse macrophages and instead is degraded by them. Studies concerning first stage uncoating of virus using sodium suramin as a host lysosome stabilizer indicated no change in viral titers and acid phosphatase particulate staining. This indicated that first stage uncoating of virus was not achieved due to lack of phagosome-lysosome fusion in the presence of suramin.

Electron micrograph studies revealed the presence of cytoplasmic cores, indicative of first stage uncoating, in both resistant and susceptible cells. Further studies concerning the second stage of uncoating indicated presence of degraded radioactive viral nucleotides in the supernatant of both mouse and rabbit peritoneal macrophages.

Autoradiography studies of infected mouse and rabbit macrophages showed that no significant viral DNA synthesis occurred in mouse cells when compared to rabbit cells. This indicated that the block of the virus replicative cycle in

mouse cells occurs early during the infection.

Evidence obtained points to the suppression of viral DNA synthesis in normal mouse macrophages as a means of expressing innate immunity against vaccinia virus. One possible mechanism for the block early in the replicative cycle of vaccinia is the presence of interferon in mouse cells.

A COMPARATIVE STUDY OF EARLY EVENTS  
IN RESISTANT AND SUSCEPTIBLE MACROPHAGES  
INFECTED WITH VACCINIA VIRUS

A Thesis

Submitted to

the Division of Biological Sciences  
Emporia State University

In Partial Fulfillment

of the Requirements for the Degree  
Master of Science

by

Fatah Kashanchi

May, 1986

## ACKNOWLEDGEMENTS

I wish to thank Vickie Clark for her technical assistance, Roger Ferguson for providing animals needed, my committee members, Dr. Edward Rowe and Dr. Richard Keeling for their time and patience. Also, Dr. Katherine Smalley for her instruction and guidance pertaining to electron microscopy.

I would like to express my warmest thanks to my parents for their support, both emotional and financial. Also my deepest appreciation to Lori Samuels for her assistance and love throughout my study.

Most important, my mentor, Dr. Helen McElree whom I greatly admire for her respect toward the field of Immunology. I am indebted for her knowledge, enthusiasm and endless support. In addition, I wish to thank her for her assistance in obtaining a special research award which supported me during my graduate work at Emporia State University.

453000

DP AUG 29 '86

Approved for Graduate Council

Approved for Major Department

*[Handwritten signature]*

## TABLE OF CONTENTS

	PAGE
LIST OF TABLES. . . . .	vi
LIST OF FIGURES . . . . .	vii
INTRODUCTION. . . . .	1
MATERIALS AND METHODS . . . . .	9
Experimental Animals. . . . .	9
Cell Culture Media. . . . .	9
Cell Culture Procedures . . . . .	9
1) Mouse Embryo Fibroblasts. . . . .	9
2) Mouse Peritoneal Macrophages. . . . .	10
3) Rabbit Peritoneal Macrophages . . . . .	11
Virus . . . . .	12
1) Propagation . . . . .	12
2) Harvesting of Virus . . . . .	13
3) Virus Assay . . . . .	13
May-Grunwald Giemsa Stain . . . . .	13
Acid Phosphatase Enzyme Assay . . . . .	14
Infection of Macrophages. . . . .	15
Electron Microscopy . . . . .	15
1) Concentration of Vaccinia Virus . . . . .	15
2) Preparation of Buffer . . . . .	16
3) Preparation of Fixative . . . . .	16
4) Simultaneous Fixation . . . . .	16
5) In situ Fixation. . . . .	16
6) Scrap Cell Fixation . . . . .	17
7) Sectioning, Staining and Observing Macrophages . . . . .	18

	PAGE
Vaccinia First Stage Uncoating. . . . .	19
Vaccinia Second Stage Uncoating . . . . .	19
1) Purification of Labelled Virus. . . . .	19
2) Second Stage Uncoating Assay. . . . .	20
Autoradiography of Macrophages. . . . .	21
RESULTS . . . . .	23
Morphology of Macrophages . . . . .	23
1) Mouse Peritoneal Macrophages. . . . .	23
2) Rabbit Peritoneal Macrophages . . . . .	23
Comparison of Viral Replication in Macrophages. .	32
First Stage Uncoating . . . . .	32
Electron Microscopy . . . . .	39
Second Stage Uncoating. . . . .	66
Autoradiography of Infected Macrophages . . . . .	66
DISCUSSION. . . . .	78
SUMMARY . . . . .	85
LITERATURE CITED. . . . .	87

## LIST OF TABLES

TABLE		PAGE
1	Second stage uncoating assay of infected rabbit macrophages up to 8 h. Four h and 8 h samples show presence of degraded viral nucleotides in the supernatant of DNase treated lysates . . . . .	68
2	Second stage uncoating assay of infected mouse macrophages up to 8 h. Both 4 h and 8 h samples treated with DNase show degraded tagged viral nucleotides in the supernatant . . . . .	68

## LIST OF FIGURES

FIGURE		PAGE
1	Uninfected normal mouse macrophages after 2 h incubation (X 400). May Grunwald-Giemsa stain. . . . .	25
2	Uninfected normal mouse macrophages after 120 h incubation (X 400). May Grunwald-Giemsa Stain . . . . .	25
3	Electron micrograph of a mouse peritoneal macrophage after 24 h incubation. Golgi apparatus and mitochondria are easily seen. (X 12,000) Uranyl acetate/lead citrate stain . . . . .	28
4	Electron micrograph of a mouse peritoneal macrophage after 24 h incubation. Cytoplasmic vesicles of varying sizes are seen. (X 36,000) Uranyl acetate/lead citrate stain. . . . .	30
5	Normal mouse peritoneal macrophage infected with vaccinia at MOI of 1.00 for 120 h. No cell clumping or death observed. (X 400) May Grunwald-Giemsa stain. . . . .	32
6	Uninfected rabbit peritoneal macrophages after 2 h incubation (X 400). May Grunwald-Giemsa stain. . . . .	32
7	Electron micrograph of a rabbit peritoneal macrophage after 24 h incubation. Several mitochondria and part of a red blood cell is seen in the left corner. (X 36,000) Uranyl acetate/lead citrate stain. . . . .	35
8	Infected rabbit peritoneal macrophages with vaccinia virus at MOI of 1.00 after 48 h incubation (X 400). May Grunwald-Giemsa stain . . . . .	37
9	Infected rabbit peritoneal macrophages with vaccinia virus at MOI of 1.00 after 120 h incubation. Typical viral induced CPE is seen (X 400). May Grunwald-Giemsa stain. . . . .	37
10	Comparison of viral titers in infected rabbit and mouse peritoneal macrophages up to 120 h. . . . .	39

11 Comparison of viral titers between suramin treated (macrophage + virus + suramin) and control (macrophage + virus) cells up to 120 h. . . . . 42

12 Uninfected normal mouse macrophages after 120 h incubation. Normal cytoplasmic granules are seen in the cytoplasm. (X 400) Acid phosphatase stain . . . . . 44

13 Vaccinia infected mouse peritoneal macrophages 120 h post infection. Presence of intense granular staining in the cytoplasm is seen. (X 400) Acid phosphatase stain . . . . . 44

14 Mouse peritoneal macrophages infected with vaccinia plus suramin 120 h post infection. Minimal amount of granular staining is seen. (X100) Acid phosphatase stain. . . . . 46

15 Electron micrograph of infected mouse peritoneal macrophages at zero min. (X 36,000) Uranyl acetate/lead citrate stain. . . . . 49

16 Electron micrograph of infected rabbit peritoneal macrophages at zero min. (X 24,000) Uranyl acetate/lead citrate stain. . . . . 49

17 Electron micrograph of infected mouse peritoneal macrophage after 30 min. Phagocytosis of a viral particle is seen. (X 24,000) Uranyl/acetate/lead citrate stain. . . . . 51

18 Electron micrograph of infected rabbit peritoneal macrophage after 30 min. Viral particles are seen both inside and outside the cell. (X 28,000) Uranyl acetate/lead citrate stain . . . . . 53

19 Electron micrograph of infected mouse peritoneal macrophage after 60 min. (X 8,000) Uranyl acetate/lead citrate stain. . . . . 55

20 Electron micrograph of infected rabbit peritoneal macrophage after 60 min. (X 24,000) Uranyl acetate/lead citrate stain. . . . . 57

21	Electron micrograph of infected mouse peritoneal macrophages after 90 min. (X 68,000) Uranyl acetate/lead citrate stain. . . . .	59
22	Electron micrograph of infected rabbit peritoneal macrophages after 90 min. (X 24,000) Uranyl acetate/lead citrate stain. . . . .	61
23	Electron micrograph of infected mouse peritoneal macrophages after 120 min. Viral core and its spikes are seen in the cytoplasm of the cell. (X 68,000) Uranyl acetate/lead citrate stain. . . . .	63
24	Electron micrograph of infected rabbit peritoneal macrophages after 120 min. Viral core is seen outside of the vacuole in the cytoplasm. (X 48,000) Uranyl acetate/lead citrate stain . . . . .	65
25	Autoradiograph of uninfected rabbit peritoneal macrophages after 8 h (X 400) . . . .	71
26	Autoradiograph of infected rabbit peritoneal macrophage at MOI of 1.00 after 8 h. Cytoplasmic silver grains are seen (X 400) . . . . .	71
27	Autoradiograph of infected rabbit peritoneal macrophages at MOI of 1.00 after 8 h. Cytoplasmic silver grains are seen (X 400) . . . . .	73
28	Autoradiograph of uninfected normal mouse peritoneal macrophages after 8 h (X 400). . . . .	75
29	Autoradiograph of infected normal mouse peritoneal macrophages after 8 h (X 400) . . . .	75
30	Comparison of viral induced DNA synthesis in normal mouse and rabbit peritoneal macrophages infected with IHD vaccinia up to 8 h.	
	CR = control rabbit (uninfected)	
	IR = infected rabbit	
	IM = infected mouse	
	CM = control mouse (uninfected) . . . . .	77

## INTRODUCTION

One of the most important roles of the immune system is defense against infection. Normally, mechanisms involved in defense are of two major kinds ; innate resistance and acquired immunity. Innate resistance is developed through inheritance and normally is passed on to new generations whereas acquired immunity develops in an individual as the result of physiological responses to an infection or immunization and is responsible for long term specific protection against subsequent infection.

Today, there is a large body of knowledge concerning acquired immunity. As a result, many of the physiological events which occur during the initial infection and remain in effect to protect the host from subsequent infection by the same etiological agent are known. However, relatively little is known about the mechanisms of innate immunity. Understanding the mechanisms which provide for innate host protection is of importance because defects in these mechanisms render the host vulnerable to infection.

Aside from ill-understood "essential factors" which make one species innately susceptible and another resistant to certain infections, a number of non-specific anti-microbial systems have been identified which are innate in the sense that they are not inherently affected by previous contact with the infectious agent. One of these is the mononuclear phagocytic cell system.

The term mononuclear phagocyte covers a group of cells

including the macrophages, found in the tissues and body cavities, and the ancestors of these cells, the monocytes in the peripheral blood. The macrophages, which play an important role in the host defense against foreign substances, are known to be derived from circulating monocytes (Hood, 1978).

Metchnikoff (1892) was the first to describe macrophages and microphages, the latter now being called polymorphonuclear leucocytes, and to recognize that these phagocytes not only serve as scavengers but also have an important function in the host defense against microorganisms. Although he worked with both macrophages and microphages, his work eventually centered on the macrophages' role in cellular immunity. Macrophages, capable of being activated to protect the host, have been recognized as an important cell line in natural host defense against a wide variety of invading microorganisms and developing neoplasms (Barrett, 1983). These cells have been shown to have extensive biosynthetic capabilities resulting in the secretion of complement components, a wide array of enzymes, interferon, lymphokines, and numerous other biologically active factors which modulate the activities of other lymphoreticular cells (Hood, 1978).

One of the major protective functions of macrophages is that of phagocytosis. Phagocytosis is a dynamic process that involves multiple steps, the cytology of which has been well described by Cohn and Wiener (1963,a) in peritoneal and liver Kupffer cells. The biochemical

correlates of the cytological events, however, are considerably less well understood. Phagocytosis can be divided into three major stages each with separate determinants (Axline, 1970). The first step, pseudopod extension and particle attachment, involves the apposition of the phagocytic cell's plasma membrane to the particle surface. It can occur even in the absence of cellular metabolism and is dependent on the surface properties of the particle and the surface properties of the cell. The second step, signal generation and signal transmission, is least understood. Before a particle can be ingested, it must bind to the surface of the phagocytic cell. Thus, it seems clear that the attachment step results in physical and/or chemical changes in the cell that trigger ingestion of the particle. The attachment step is followed by the ingestion phase, which involves invagination of the surface membrane to surround the particle with subsequent interiorization of the complex.

Tsan and Berlin (1971), using alveolar macrophages, showed that between 30 to 50 percent of the surface membrane was internalized during phagocytosis. They also established that membrane transport carriers for amino acids and purine bases were completely unchanged after internalization of a large portion of the membrane.

The ultimate control or eradication of organisms being phagocytized is largely dependent upon the macrophages becoming activated. Cohn and Wiener (1963,b) studied the

cytological features of activated macrophages and found large numbers of perinuclear inclusions which they biochemically characterized as lysosomes. When lysates of rabbit alveolar macrophages were separated by differential centrifugation increased amounts of acid phosphatase, lysozyme, acid ribonuclease and beta-glucuronidase activity were found. Schnyder and Baggiolini (1978) using Brewer's thioglycollate (TA) medium found more protein, lactate dehydrogenase, lysosomal hydrolases (beta-glucuronidase, N-acetyl-Beta-glucosaminidase and acid phosphatase) when using isopycnic centrifugation to analyze lysosomal contents of mouse peritoneal macrophages.

Activated macrophages have been shown to eliminate intracellular parasites such as Mycobacterium tuberculosis, Trypanosoma cruzi and vaccinia virus. In vivo eradication in almost all of these organisms requires the differentiation of specific T-cell immunity and further production of activated macrophages. Vaccinia infection of macrophages in vitro provides an excellent model for further study of mechanisms operating to foster or suppress parasite survival at the cell level. This is due to the fact that vaccinia virus replicates in normal rabbit macrophages but not in normal mouse macrophages.

According to Joklik (1966), vaccinia virus is a member of the vaccinia subgroup which is closely related antigenically to variola, the virus responsible for small pox disease. Five to ten percent of its genome is made up of single-stranded DNA with the rest being double-stranded

of single-stranded DNA with the rest being double-stranded DNA encircled by a filamentous proteinaceous membrane. This complex is usually called a "core" and is surrounded by two lateral bodies. Finally, the core and lateral bodies are enveloped by a trilaminar or "unit" membrane. Dales and Mosbach (1968) analysed the outer membrane of vaccinia and discovered that chemically it is composed of protein and lipid, of which lecithin was the predominant phospholipid. They also concluded that choline was the specific precursor of both vaccinia and host cell (L-cell) phospholipid.

Easterbrook (1966) used a nonionic detergent and mercaptoethanol to control the process of degradation of vaccinia virus. He found that the outer coat is first removed (first stage uncoating), then the so-called lateral bodies are removed, and at last the nucleocapsid or core was emptied (second stage uncoating) releasing genomic DNA into the cytoplasm. The entire process took between 30 minutes to 1.5 hours depending on the multiplicity of infectious virus. Joklik (1964) went on to describe that viral genomes which have been damaged by ultraviolet light fail to induce an enzyme which is responsible for second stage uncoating and hence are unable to release their DNA to initiate a new cycle of viral replication. In contrast, heat-denatured or antibody neutralized vaccinia enters the phagocytic vacuole of the cell but is unable to escape from it. Subsequently, the phagocytic vacuole fuses with primary and secondary lysosomes and degrades the virus.

Macrophages have been shown to play a significant role in the pathogenesis of infection by vaccinia virus. Greer (1973) found that macrophages obtained from rabbits immunized with vaccinia virus failed to support replication of the virus. Based on electron micrograph studies, immune and non-immune macrophages took up equal amounts of virus. Along the same lines Buchmeier, et al, (1979) found similar results and also detected first and second stage uncoating as well as DNA synthesis in both immune and non-immune rabbit macrophages. However, a major difference was found between activated and normal macrophages in the assembly of progeny virus in the infected cells. Very few activated macrophages were found to contain immature or mature virions. This was in marked contrast to normal macrophages where the majority of the cells contained mature virus particles.

On the other hand, studies concerning the mechanisms of restriction of pox viruses in peritoneal exudate cells from infected mice have produced conflicting results. Schultz (1966) examined vaccinia cytopathic effects in both mouse and rabbit peritoneal macrophages. He concluded that the mechanism of resistance of mouse macrophages and susceptibility of rabbit macrophages were largely due to the presence of hydrolytic enzymes in the mouse macrophage which denatured the viral uncoating component and led to the degradation of the virus particle within phagocytic vacuole. Using electron microscope technique, Clark (1984) investigated activated and normal mouse macrophages infected

with vaccinia virus. Using high viral titer, she found that thioglycollate elicited mouse macrophages allowed first stage uncoating, but normal mouse macrophages did not. Yet Ward (1980), using only thioglycollate elicited mouse macrophages, concluded that naked viral DNA was present within the normal mouse cells, which is indicative of second stage uncoating. In addition, autoradiograph studies indicated that no apparent viral DNA synthesis occurred. Hence, it was suggested that the presence of free viral DNA was probably due to viral degradation and not evidence of a replicative cycle. It is also of interest to note that all of the above individuals used IHD strain of vaccinia virus and an inbred strain of white albino mice.

A different analysis was presented by Silverstein (1970) on macrophage-vaccinia interaction. In an electron microscope study, in conjunction with biochemical experiments using <sup>3</sup>H-thymidine labeled IDH strain of vaccinia, he showed that first stage uncoating took place, but no DNA was released from the core. This resulted in a "toxic" effect of vaccinia virus on macrophages for which the molecular basis was unknown. However, it was speculated that proteins within the virus particle were released into the cytoplasmic matrix possibly altering cell membrane permeability and triggering the primary toxic event. Unfortunately, data obtained from that study is hard to evaluate because the experimental conditions, as well as the strain of mice used, were not described.

Because the macrophages of some animal species appear to have a role in resistance of that species to pox virus, demonstration of the mechanisms by which the macrophage restricts virus replication could contribute to our understanding of host innate resistance. The present study was undertaken to examine the mechanism(s) which allow normal mouse peritoneal macrophages to suppress vaccinia replication and to determine the fate of the virus. In particular, answers were sought to the following questions: Does first or second stage uncoating take place in mouse macrophages? Does viral DNA synthesis occur in mouse macrophages? To answer the above questions, radioactively labeled virus, autoradiography of infected cells and electron micrograph studies were applied.

## MATERIALS AND METHODS

### Experimental Animals

Young adult albino mice of both sexes were grown in the Emporia State University biology animal room. Six to eight month New Zealand white rabbits were obtained locally for use in the experimental procedures.

### Cell Culture Media

All cell types used in this study were cultured in Eagles Minimal Essential Medium (MEM) containing l-glutamine plus combiotic (Penicillin G, 200 units per ml, and Streptomycin, 100 units per ml) and sterile fetal calf serum (FCS) at a final concentration of 10 percent. MEM plus two units of heparin per ml was used for the harvesting of both mouse and rabbit macrophages. Sterile 1.5% or 7.5% sodium bicarbonate was used to adjust the pH of the culture when necessary.

A stock solution of dialyzed and lyophilized trypsin (Sigma, S.P. = 12,400 BAEE units/mg protein) 5% w/v was prepared in Dulbecco's phosphate buffer solution, without Mg<sup>2+</sup> or Ca<sup>2+</sup> (DPBS), membrane filtered, and stored at 0 C. EDTA was used to chelate Ca<sup>2+</sup>, which interferes with trypsin action. The trypsinizing solution contained the final concentration of 0.25% trypsin and 0.02% EDTA.

### Cell Culture Procedures

#### 1) Mouse Fibroblast Cultures

Pregnant mice were sacrificed by cervical dislocation on the tenth to sixteenth day of gestation. The abdominal hair and skin were saturated with ethyl alcohol and the

ventral skin laid back. The abdomen was opened to expose the uterus and the embryos were removed and placed in a sterile petri dish containing sterile MEM plus combiotic. The embryos were decapitated, eviscerated, and transferred to another petri dish containing fresh sterile MEM. After mincing with a sterile disposable scapel, the embryo fragments (about 1 cubic millimeter) were placed in a 50 ml trypsinizing solution consisting of 47.00 ml of MEM plus 2.5 ml of 0.25% trypsin and 0.5 ml of 0.02% EDTA. The cells were dispersed for 2 h at room temperature by stirring on a magnetic stirrer. At the end of 2 h, 5 ml of sterile FCS were added to the flask to stop the action of trypsin. The cells were poured through a sterile cheese cloth filter to remove debris and cell clumps. The resultant cell suspension was washed two times with DPBS at 2000 rpm for 10 min and counted with an improved Neubauer hemocytometer. Cells were adjusted to  $2.5 \times 10^6$  cells per ml with MEM plus 15% FCS and combiotic. Fifty to sixty ml of the adjusted cell suspension were planted in Roux flasks and allowed to monolayer at 37 C for 36 to 48 h. Remaining cells were further diluted to  $6.25 \times 10^5$  cells per ml with MEM plus combiotic and planted in eight tissue culture flasks, each containing 5 ml of cell suspension. Cell monolayers were used for viral titration.

## 2) Mouse Peritoneal Macrophages

Adult mice were sacrificed by cervical dislocation throughout the experiments. Resident mouse macrophages,

which are cells obtained without a known inflammatory agent, were harvested by injecting 3 to 4 ml of MEM plus heparin into the peritoneal cavity. Retrieved fluid usually had a cell count between  $3 \times 10^6$  to  $5 \times 10^6$  cells/ml, of which 80 to 90 percent were macrophages.

The cell suspension was washed twice in cold DPBS, counted, and adjusted to a concentration of  $3.0$  to  $3.5 \times 10^6$  cells per ml. Cells were then planted in either flying coverslip tubes or plastic cell culture flasks in 1 ml or 5 ml amounts respectively and allowed to settle for 2 hr at 37 C. The nonadherent cells were then washed away with warm DPBS and replaced with MEM plus combiotic and 10% FCS.

### 3) Rabbit Peritoneal Macrophages

Healthy white rabbits 36 months old were given a 50 ml intraperitoneal injection of sterile mineral oil (Topco heavy duty) which resulted in a population of elicited macrophages. After 3 days animals were sacrificed by air embolism. One hundred ml of MEM plus heparin and combiotic were injected into the peritoneal cavity. The abdomen was gently massaged for approximately 1 min, and then aseptically opened. The fluid was removed with a sterile 100 ml volumetric pipette using gentle suction and placed in a separatory funnel. The funnel was placed at 4 C for 15 minutes to allow separation of the aqueous and oil phases. The aqueous phase, which contained nucleated cells, was drained into 50 ml centrifuge tubes and centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted and the cells washed twice with cold DPBS. The cells were then

counted and adjusted to  $3 \times 10^6$  cells per ml with MEM plus 10% FCS. The cells, which normally yield on the order of  $200 \times 10^6$ , were obtained from induced animals. After settling for 2 h at 37 C, the nonadherent cells were removed by washing twice with warm DPBS and replaced with fresh MEM plus combiotic and 10% FCS.

## Virus

### 1) Propagation

The IHD strain of vaccinia virus used in this study was originally obtained from the American Type Culture Collection.

Roux flasks, containing 50 ml of embryonic cell suspension, were incubated at 37 C for 36-48 h until the cells were sparsely monolayered. The medium was removed, the cell monolayer washed two times with D-PBS and inoculated with 10 ml of MEM consisting of 2% FCS and 10 plaque forming units (pfu) of IHD vaccinia per ml. The inoculum was allowed to remain in contact with monolayer for 2 h. During this period flasks were gently agitated every 5 min to facilitate adsorption. The infecting medium, along with any unadsorbed virus, was removed by two washings with warm D-PBS and replaced with 50 to 60 ml of MEM containing 10% FCS. The cells were incubated at 37 C until extensive viral-induced cell destruction was observed.

Radioactively labeled virus was prepared by infecting mouse embryo monolayers. After viral adsorption, viral DNA was labeled with 60 ml of MEM containing 3H-thymidine (ICN

Radiochemicals) at a final concentration of 10 uci per ml. Monolayers were incubated at 37 C for 48 h until cell damage was observed.

## 2) Harvesting of Virus

Harvesting the virus consisted of subjecting the infected cell cultures to three cycles of freeze-thaw to release intracellular virus. Cell debris was removed by centrifugation at 2500 rpm for 10 minutes. The resulting supernatant was pooled, aliquoted in 5 ml amounts, and stored at -10 C until use.

## 3) Virus Assay

Ten-fold dilutions of stock virus to be titered were prepared in MEM with 1% FCS. One ml of the four highest dilutions was inoculated into culture flasks containing an almost monolayered mouse embryo fibroblasts. After incubation at 37 C for 2 h with mild agitation every 5 min, the MEM containing unadsorbed virus was removed, washed with warm D-PBS, followed by the addition of MEM plus combiotic and 10% FCS. The flasks were incubated at 37 C until plaque formation was evident. The medium was removed and the monolayers stained with Gram's crystal violet diluted 1:10 with water and the plaques were counted. The final activity of virus was expressed as plaque forming units (pfu) per ml.

## May-Grunwald Giemsa Stain

Coverslips were washed, air dried, and fixed in absolute methanol for 5 min. After fixation, they were stained in May-Grunwald stain for 9 min and Giemsa stain for

14 min. The coverslips were rinsed in acetone, then in acetone-xylene mixture (1:1) and cleared in xylene for 10 min. Finally, coverslips were mounted cell side down in Kleermount xylene solution on glass slides.

#### Acid-Phosphatase Enzyme Assay

Coverslips were initially processed in one of three ways. For a positive control, 10% cold neutral formalin was added to coverslips to facilitate an artificial activation of lysosomes (Milligan 1983). For a negative control, sodium glycerophosphate of substrate solution was omitted.

Coverslips were placed in warm substrate solution and incubated in a water bath for 2 h at 37 C. The substrate solution was prepared by adding the following to 175 ml of deionized water: glacial acetic acid, 0.225 ml; sodium acetate (NaAc: 3HOH), 1.19 g; lead nitrate, 0.3 g; sodium glycerophosphate, 0.75 g, which was added slowly with stirring in 25 ml of HOH. The solution was then incubated overnight at 37 C, decanted and filtered through a millipore filter. The volume was increased to 250 ml with water and pH was adjusted to 5.5 with 0.1 N HCl.

After incubation in the substrate solution, the coverslips were rinsed in 0.5% acetic acid for 30 sec, rinsed twice with distilled HOH, stained in dilute ammonium sulfide (10%) for 5 min and washed in water for 3 min. Dehydration was achieved through alcohols (40%, 60%, 80%, 100%) and the coverslips were mounted in phosphate-buffered glycerol (9:1) on glass slides.

## Infection of Macrophages

After 24 h incubation, the culture medium was removed and cell monolayers were washed twice with D-PBS. One coverslip was stained with May-Grunwald Giemsa to detect overall cytological appearance before running the experiment. If cells appeared healthy, they were divided into two groups, one to be infected and the other to serve as control. Tubes to be infected received 1 ml of infecting medium consisting of MEM plus  $3 \times 10^6$  pfu/ml vaccinia virus. The control tubes received only MEM plus 3% FCS. After two hours of adsorption at 37 C, the supernatant was removed and replaced with culture medium. Zero time samples for viral titration were obtained by freeze-thawing two tubes. At 24 h intervals through a period of 120 h, duplicate samples of infected tubes were freeze-thawed and titered on mouse embryo fibroblasts. At the same time coverslips from control and infected systems were stained with May Grunwald-Giemsa or acid phosphatase.

## Electron Microscopy

### 1) Concentration of Vaccinia Virus

Infection of mouse and rabbit macrophages for electron microscopic observation required the vaccinia virus to have a high titer. In order to concentrate the virus, the viral suspension was centrifuged for 90 min at 4 C at 25,000 rpm using a fixed angle head. The virus was then resuspended in the proper volume of sterile medium to give the desired titer. Viral clumps were dispersed by sonification in a Raytheon Sonic Oscillator at 0.6 amperes for five cycles of

fifteen seconds each.

## 2) Preparation of Buffer

Mellonig's phosphate buffer was prepared as described by Hayat (1970). Initially, three solutions were prepared: solution A, 2.26% monobasic sodium phosphate; solution B, 2.52% sodium hydroxide; and solution C, 5.40% D-glucose. Fourth solution D was prepared by adding 41.5 ml of solution A to 8.5 ml of solution B. The final buffer was prepared by adding 5 ml of solution C to 45 ml of solution D. The resultant buffer was adjusted to a pH of 6.5 before use.

## 3) Preparation of Fixatives

Two percent OSO<sub>4</sub> solutions were prepared by 1:1 dilution of 4% OSO<sub>4</sub> with buffer. Two and one-half percent gluteraldehyde solution was prepared by diluting a 25% stock solution. In both cases, Mellonig's buffer was used as a diluent. Point five percent uranyl acetate in 70% ethanol was used for both in situ and scraped cell post fixations.

## 4) Simultaneous Fixation

Pre-cooled solutions of glutaraldehyde and OSO<sub>4</sub> were combined at 4 C just prior to use. The resultant mixture consisted of two parts 2% OSO<sub>4</sub> and one part 2.5% glutaraldehyde in the buffer. A slightly yellow color resulted from the mixture.

## 5) In situ Fixation

The monolayer, which had been infected with a 10:1 virus:cell ratio, was pre-cooled to 4 C in an ice bath for 10 min. Culture medium was poured off and cells were washed

four times with Mellonig's buffer. Four ml of mixed fixative was added for 90 min at 4 C. Next, the sample was washed three times with cold buffer, twice with 0.15 M NaCl and post-fixed with 0.5% uranyl acetate for 30 min at 4 C. Dehydration was initiated with 5 min of 70% ethanol followed by three 10 min washes with absolute ethanol. The absolute ethanol was removed from the flask as completely as possible. The sample was embedded in a mixture of epon araldite epoxy resin. The resin was polymerized by an overnight incubation at 40 C followed by a 48 h incubation at 80 C. After polymerization, resin containing macrophages were peeled off from the plastic surface of the culture flask and cut into small pieces for sectioning.

#### 6) Scraped Cell Fixation

After adsorption of virus, the macrophage monolayer was pre-cooled to 4 C and washed four times with buffer. Next, 4 ml of mixed fixative was added for 60 min at 4 C. The cells were then gently scraped from the surface of culture flask with a rubber policeman. The cell suspension was transferred to a conical tube and centrifuged at 1800 rpm for 7 min. The supernatant was removed with a pasteur pipette, the pellet was chilled on ice, suspended in 4 ml of fresh aliquot of mixed fixative, and kept on ice for 30 min, followed by two washes with cold buffer. Post-fixation was carried out for 30 min using 0.5% uranyl acetate in 70% ethanol. Cells were washed twice in warm buffer and then warm, melted 2% nobel agar was added to the cell pellet. After the agar had hardened, it was cut into 1 cubic

millimeter blocks where clumps of cells were apparent.

The macrophages were dehydrated and infiltrated at room temperature. The cells were first placed in 40% ethanol for two washes of 5 min each. Successive dehydrations were for 10 min each in 60%, 80%, and 95% ethanol. The final three washes were in 100% ethanol for 10 min each. Infiltration was initiated by three washes for 10 min 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 1 h an equal volume of working mixture was again added to the mixture already present for approximately 3 h. Embedding was achieved by adding pure working mixture to plastic beam capsules along with the tissue. The blocks were polymerized by an overnight incubation in a 35 C oven followed by a 48 h incubation at 80 C.

#### 7) Sectioning, Staining and Observing Macrophages

After complete polymerization, cells were sectioned on an LKB ultramicrotome. The sections were picked up on 300 mesh copper grids, dried and stained with 2% aqueous uranyl acetate for 15 min followed by 2 min of Reynold's lead citrate. After they were dry, the grids were observed and photographed under the Hitachi Electron Microscope (Model HS-8).

Photographic negatives were developed for 4 min in D-19 developer, rinsed with tap water for 2 min, fixed for 10 min, and then rinsed in tap water for 30 min. The negatives were allowed to dry overnight whereupon they

were enlarged and developed on poly-contrast II kodak paper.

### Vaccinia First Stage Uncoating

In order to detect whether first stage uncoating took place as a result of lysosomal activation, 50 ug/ml of sodium suramin (Mobay Chemical Corporation) was used to stabilize the membranes of the mouse macrophage lysosomes. Initially three sets of tubes were used: a negative control set where cells were not infected with vaccinia, an experimental set where cells were infected with vaccinia at a 1:1 ratio plus 50 ug/ml of suramin, and a positive control where cells were only infected with virus alone. At 24 h intervals up to 120 h, cells were stained by a histochemical stain specific for acid phosphatase enzymes and titrated on mouse embryo fibroblasts.

To insure that sodium suramin did not have any effect on the virus, a sample consisting of 50 ug/ml suramin plus 1 ml of  $4 \times 10^6$  pfu virus was assayed on mouse embryo fibroblasts. This was to establish viability and infectivity of viral particles when running the experimental set where virus plus suramin was added to mouse peritoneal macrophages.

### Vaccinia Second Stage Uncoating

#### 1) Purification of Labelled Virus

Adherent mouse embryo fibroblasts were infected with vaccinia virus and DNA was labelled with <sup>3</sup>H-thymidine. After viral multiplication, the cell monolayers were subjected to three cycles of freeze-thaw and centrifugation at 2500 rpm to deposit unwanted cell debris. The pooled

supernatant which contained the labelled virus was layered on 36% sucrose (w/v) in distilled HOH and centrifuged at 25000 rpm for 100 min. This allowed formation of a pellet of labelled virus plus cellular DNA. In order to eliminate cellular DNA, the pellet was resuspended in 5 ml of PBS plus DNAase I (200 ug/ml) in 50 mM Mg<sup>2+</sup> for 30 min at 37 C. The resuspended pellet was sonicated at 0.6 amps for 5 cycles of 15 sec each to eliminate viral clumping. Next, the suspension was layered on 36% sucrose and centrifuged at 25,000 rpm for 100 min. The pellet containing the virus was resuspended in 10 ml of MEM plus combiotic and two 0.1 ml samples were taken to determine the amount of radioactivity as well as the final concentration of virus.

## 2) Second Stage Uncoating Assay

3-H DNA labelled virus was adsorbed to macrophages (10:1 ratio) at 4 C which allowed for viral attachment but not penetration into the cell (Joklik, 1963). The unadsorbed virus was removed and the virus-cell complexes were warmed to 37 C, initiating a synchronous wave of viral penetration. At 30 min, 4 h and 8 h after warming, the cells were scraped from the culture flask and centrifuged to deposit cells. The cell pellet was processed by resuspending it in 2.2 ml of a solution of 0.01 M sodium phosphate and 0.01 M MgCl at pH 7. Next, cells were sonicated followed by two cycles of freeze-thaw to release free viral DNA present from the cytoplasmic matrix of the cells. A total count was obtained at this point by taking

0.2 ml quantities from each sample and counting them on a LKB liquid scintillation counter. One half of the remaining sample was treated with TCA (0.2 ml of 50% stock TCA) and the other half with TCA plus 500 ug/ml of DNAase I, which then was incubated at 37 C for 30 min. Both samples were held overnight at 4 C and centrifuged the next day to separate supernatant from TCA precipitate. The percentage of radioactivity in each sample was determined by dividing experimental counts over total count times one hundred.

### Autoradiography of Macrophages

Macrophages were harvested from both rabbits and mice and planted at  $3 \times 10^6$  cells/ml in coverslip tubes. The macrophages were allowed to settle for 24 h and infected according to the established procedure. After 2 h of adsorption, the macrophage monolayers were washed with D-PBS and Eagle's MEM with 2% serum plus 5 uc/ml of 3-H thymidine (specific activity 6.7 ci/mM) was added. After 4 h incubation at 37 C with the isotope, the cells were rinsed 3 times with D-PBS, dried and fixed with absolute methanol for 10 min. The coverslips were mounted cell side up using Permount mounting medium. Next, slides were dipped in a chrom-alum solution (1% gelatin, 0.1% chrom-alum) and allowed to dry for 30 min (Shedani, 1980).

Nuclear tract emulsion-2 (Eastman-Kodak) was warmed in a 50 C water bath located in a 20 C dark room where the rest of the procedure was carried out. When the emulsion was liquified, it was transferred onto a pre-warmed slide with a pasteur pipette. The slides were held in a vertical

position for 30 min to allow excess emulsion to run off and then placed in light-tight boxes containing a dehumidifier and stored at 4 C for 9 days.

At the end of the exposure period, the slides were developed in D-19 solution for 10 min, washed in several changes of water for 30 min, and fixed for 10 min. Washed slides were stained in 1% toluidine blue solution for 2 min, destained in 70% ethanol until cells were of a desired shade, then dehydrated by two changes of 100% n-butanol. Finally, cells were cleared in three changes of xylol and observed microscopically.

## RESULTS

### Morphology of Macrophages

#### (1) Mouse Peritoneal Macrophages

Normal peritoneal mouse macrophages which were planted and observed on stained coverslips, appeared as rounded cells with an indented, kidney-shaped nucleus (Fig. 1). Throughout a 120 h in vitro cultivation, cell diameters increased either by uniform cytoplasmic spreading or by extensions of pseudopods (Fig. 2). An ultrastructural study of these cells revealed typical cell organelles with an abundance of Golgi structures and mitochondria (Fig. 3). Perhaps the most striking feature of mouse macrophage cytoplasm was the presence of large numbers of small vesicles concentrated in the centrosomal region but also present in peripheral zones (Fig. 4). These vesicular elements appear to be of three general types: a small number of vesicles showing surface spikes typical of so-called coated vesicles; a moderate number of slightly larger vesicles with an electron lucent content; and a large number of vesicles varying in size and containing material with a density similar to, or slightly greater than, that of the cytoplasmic matrix.

Macrophages infected with vaccinia at  $3 \times 10^6$  pfu per ml exhibited a morphologic progression similar to that of uninfected macrophages, with no evidence of cell clumping or damage. After 120 h in culture, the cell morphology was identical to that of uninfected macrophages (Fig. 5).

#### (2) Rabbit Peritoneal Macrophages

Figure 1. Uninfected normal mouse macrophages after 2 h incubation (X 400). May Grunwald-Giemsa stain.

Figure 2. Uninfected normal mouse macrophages after 120 h incubation (X 400). May Grunwald-Giemsa stain.



Figure 3. Electron micrograph of a mouse peritoneal macrophage after 24 h incubation. Golgi apparatus and mitochondria are easily seen. (X 12,000) Uranyl acetate/lead citrate stain.

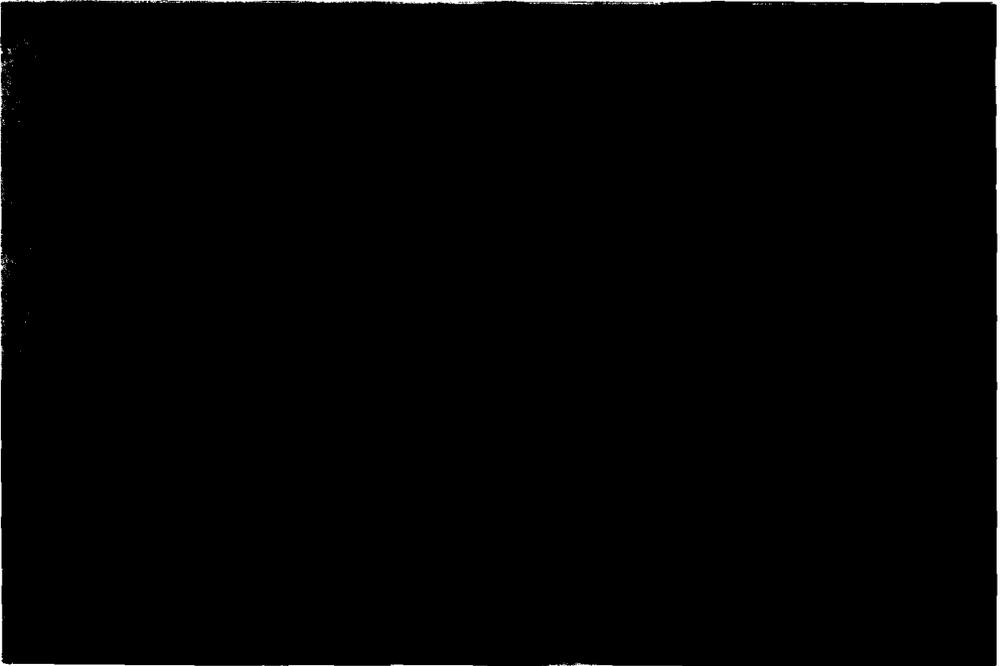
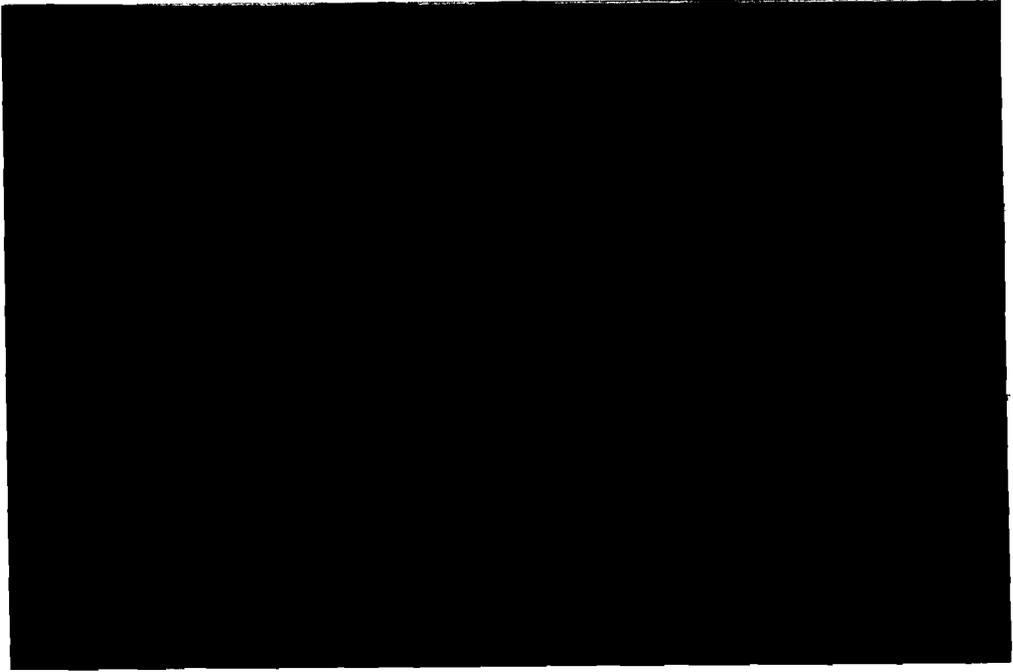


Figure 4. Electron micrograph of a mouse peritoneal macrophage after 24 h incubation. Cytoplasmic vesicles of varying sizes are seen. (X 36,000) Uranyl acetate/lead citrate stain.



Figure 5. Normal mouse peritoneal macrophage infected with vaccinia at MOI of 1.00 for 120 h. No cell clumping or death is observed. (X 400) May Grunwald-Giemsa stain.

Figure 6. Uninfected rabbit peritoneal macrophages after 2 h incubation (X 400). May Grunwald-Giemsa stain.



Stimulated rabbit peritoneal exudate, when stained after settling and washing, appeared as rounded, moderately vacuolated cells of varying sizes (Fig. 6). At the ultrastructural level rabbit macrophages always appeared to have fewer cytoplasmic vesicles as compared to mouse macrophages, but several mitochondria were seen in the peripheral cytoplasm. The cytoplasmic matrix was of moderate density and had an amorphous or microgranular appearance (Fig. 7). Macrophages infected at  $3 \times 10^6$  pfu per ml exhibited a great tendency to clump and some evidence of cellular damage was observable 48 h post-infection (Fig. 8). By 120 h there was a widespread viral toxic effect which resulted in cell damage and death (Fig. 9).

#### Comparison of Viral Replication in Macrophages

Both rabbit and mouse peritoneal macrophages were infected with vaccinia at a 1:1 viral-cell ratio. As mentioned previously, no cytopathogenic effects were seen in mouse cells throughout 120 h. Also, viral titrations at 24 h intervals indicated a marked decrease in the number of infectious virions. However, a progressive cell death as well as an increase in viral titers were observed with rabbit peritoneal macrophages (Fig. 10). This clearly established a demonstrable viral blockage in mouse cells as compared to viral multiplication in rabbit cells.

#### First Stage Uncoating

Suramin treated mouse macrophages were cultured for 120 h. Freeze-thawed samples were taken every 24 h to detect the

Figure 7. Electron micrograph of a rabbit peritoneal macrophage after 24 h incubation. Several mitochondria and part of a red blood cell are seen in the left corner. (X 36,000) Uranyl acetate/lead citrate stain.

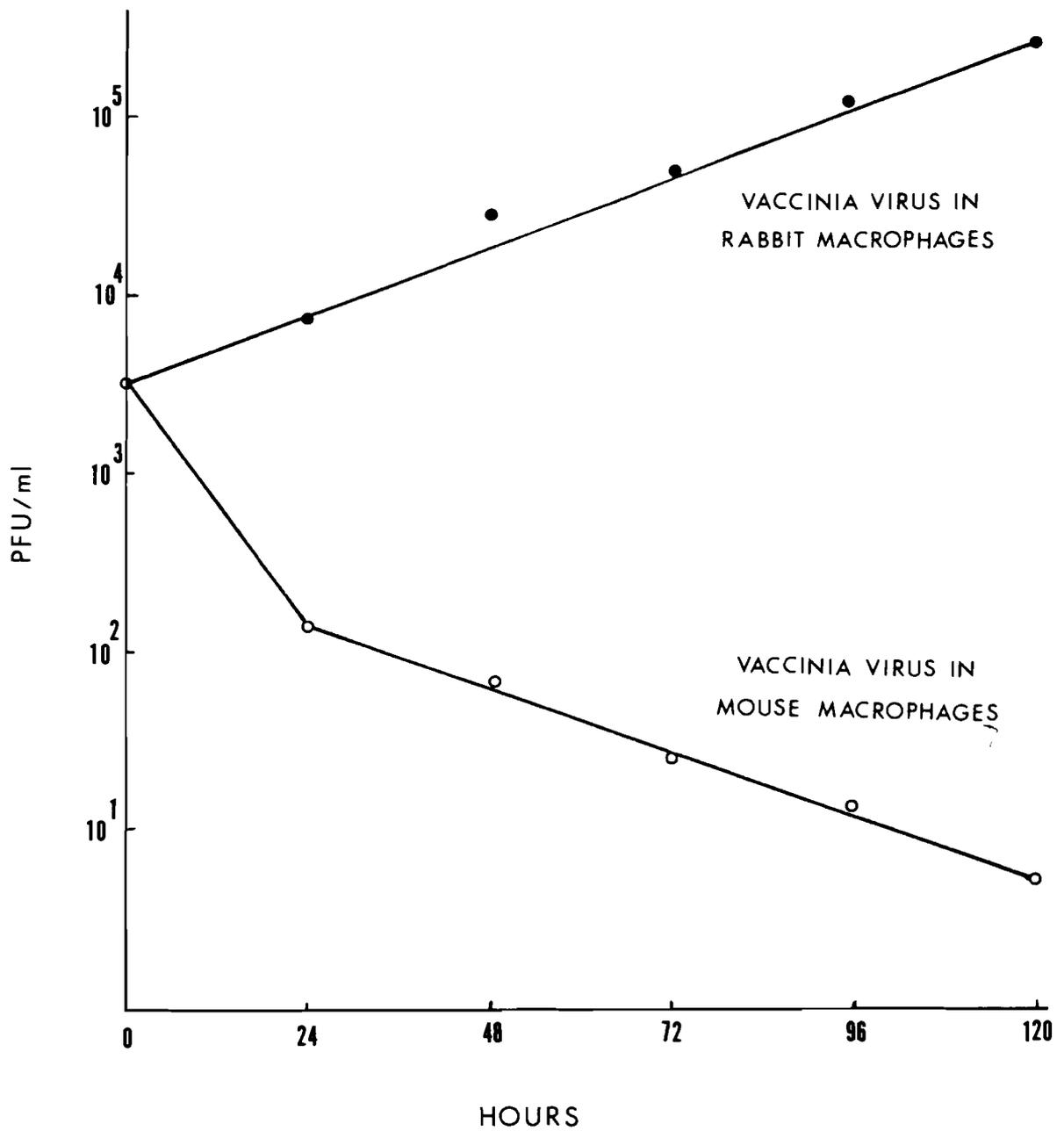


Figure 8. Infected rabbit peritoneal macrophages with vaccinia virus at MOI of 1.00 after 48 h incubation. (X 400) May Grunwald-Giemsa stain.

Figure 9. Infected rabbit peritoneal macrophages with vaccinia virus at MOI of 1.00 after 120 h incubation. Typical viral induced CPE is seen. (X 400) May Grunwald-Giemsa stain.



Figure 10. Comparison of viral titers in infected rabbit and mouse peritoneal macrophages up to 120 h.



number of remaining infective virus. Results indicated that suramin treated cells showed no dramatic decrease or increase in number of infectious virus (Fig. 11). However, untreated control cells had a dramatic decrease in number of infectious viral particles.

In order to determine enzyme activation, three sets of tubes were used: a negative control, a positive control, and an experimental set. All sets were treated with a histochemical stain specific for acid phosphatase enzymes. Uninfected negative control cultures had normal cytoplasmic staining compared to positive control cultures where intense granular staining in the cytoplasm was observed (Fig. 12, 13). However, the experimental set, where cells infected with virus plus suramin exhibited only a minimal amount of lysosomal activation and the amount of particulate staining did not increase with time (Fig. 14).

Histochemical observations together with viral titers from suramin treated cells indicated existence of undamaged viral particles and a decrease in cellular acid phosphatase enzymes. Therefore, it was concluded that first stage uncoating of vaccinia virus was possibly due to lysosomal activation of mouse peritoneal macrophages.

### Electron Microscopy

The electron microscope was also utilized to study the early intracellular fate of vaccinia within both mouse and rabbit macrophages. Initially, adsorption was allowed to take place at 4 C for 90 min and subsequent penetration was initiated by warming the cultures to 37 C.

Figure 11. Comparison of viral titers between suramin treated (macrophage + virus + suramin) and control (macrophage + virus) cells up to 120 h.

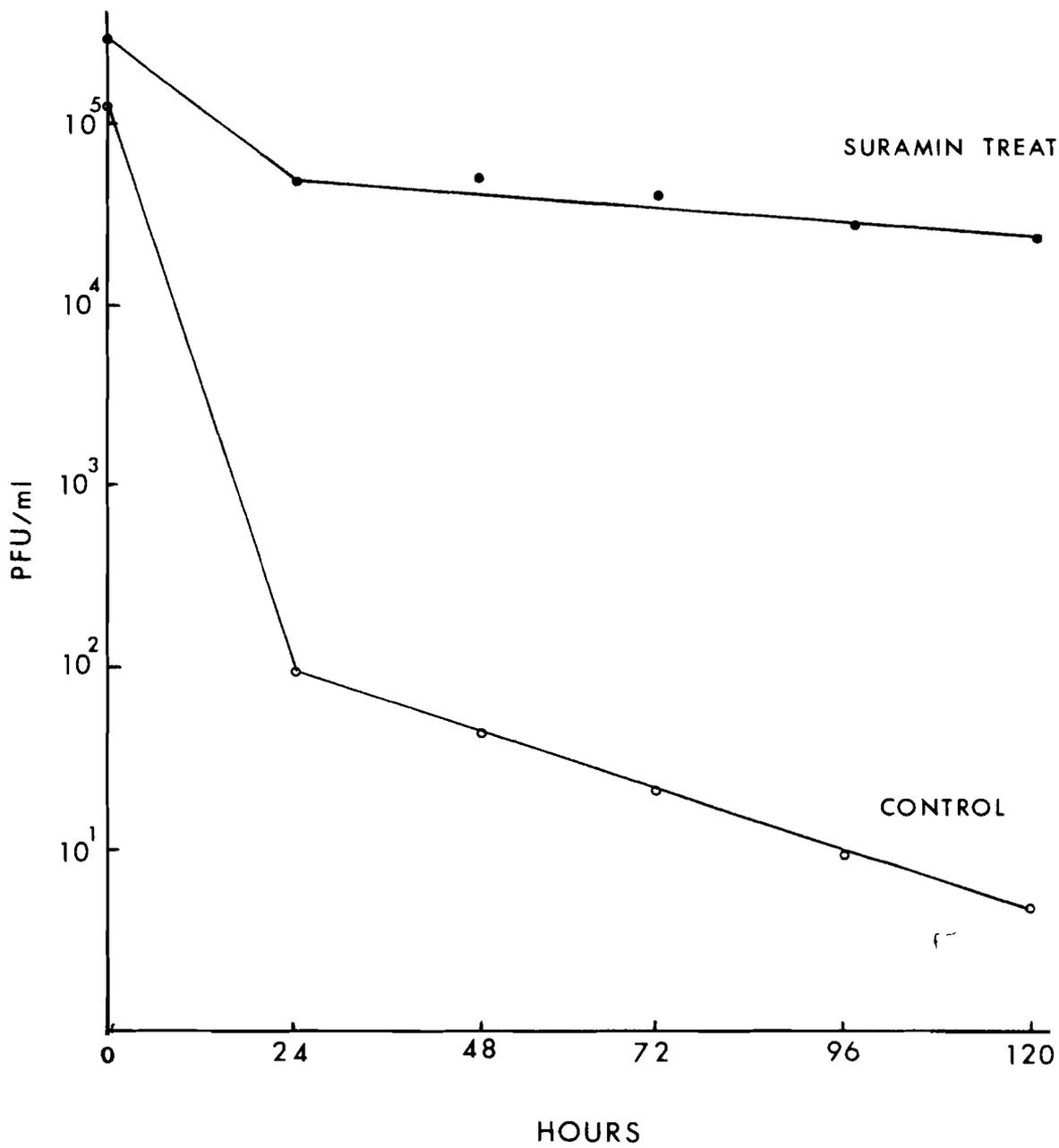


Figure 13. Uninfected normal mouse macrophages after 120 h incubation. Normal cytoplasmic granules are seen in the cytoplasm. (X 400) Acid phosphatase stain.

Figure 12. Vaccinia infected mouse peritoneal macrophages 120 h post infection. Presence of intense granular staining in the cytoplasm. (X 400) Acid Phosphatase stain.

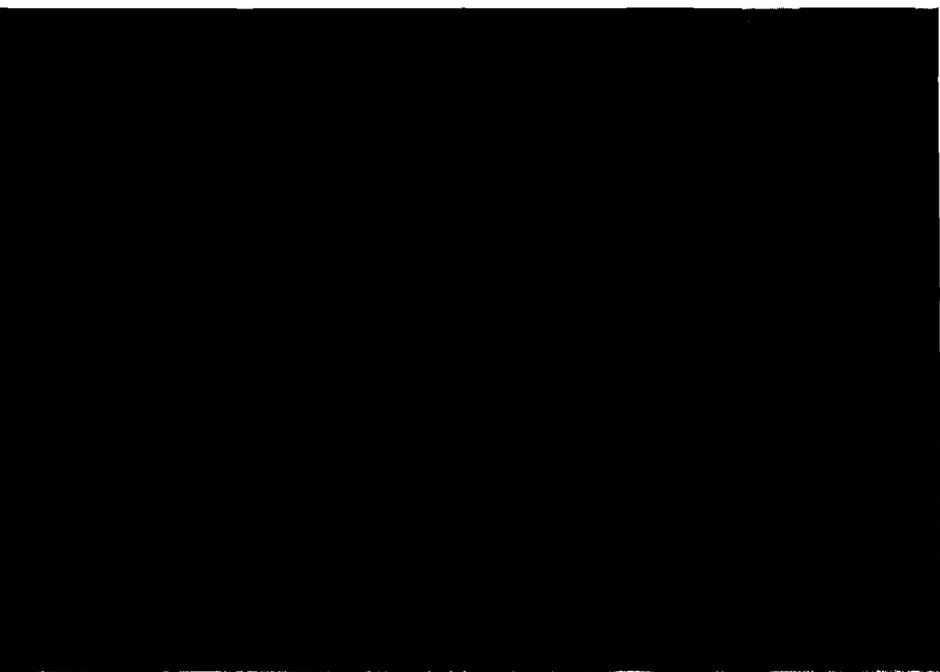


Figure 14. Mouse peritoneal macrophages infected with vaccinia plus suramin 120 h post infection. Minimal amount of granular staining is seen. (X 100) Acid phosphatase stain.



Macrophages infected with virus were processed for electron microscopy at 0, 30, 60, 90, and 120 min after initial adsorption. At each time interval, five cell blocks were sectioned, stained, and observed under the electron microscope. This insured that the same cell was not examined twice.

Zero min samples revealed attachment of virus to macrophages (Fig. 15, 16). Thirty min and 60 min samples showed both phagocytized and non-phagocytized particles (Fig. 17, 18, 19, 20). The observation implied that the concentration of infectious units did not provide a measure of the total virions participating. Ninety min samples of both animals showed the highest number of phagocytized virus (Fig. 21, 22). In general, the virus contained within the phagosome up to 90 min had the typical mature morphology of vaccinia virus with its internal double concave plate.

Observations of cell sections at 120 min revealed partial dissolution of phagocytic vacuolar walls and presence of released viral cores into the cytoplasm of infected cells (Fig. 23, 24). Cytoplasmic cores clearly established first stage uncoating of vaccinia virus in both resistant mouse and susceptible rabbit macrophages. The surface of the cores was covered by fine, short projections each approximately 200 A long. The inner portion of cores contained filamentous material which is believed to be viral nucleic acid.

#### Second Stage Uncoating

The second stage uncoating of virus was detected by use

Figure 15. Electron micrograph of infected mouse peritoneal macrophages at zero min. (X 36,000) Uranyl acetate/lead citrate stain.

Figure 16. Electron micrograph of infected rabbit peritoneal macrophages at zero min. (X 24,000) Uranyl acetate/lead citrate stain.

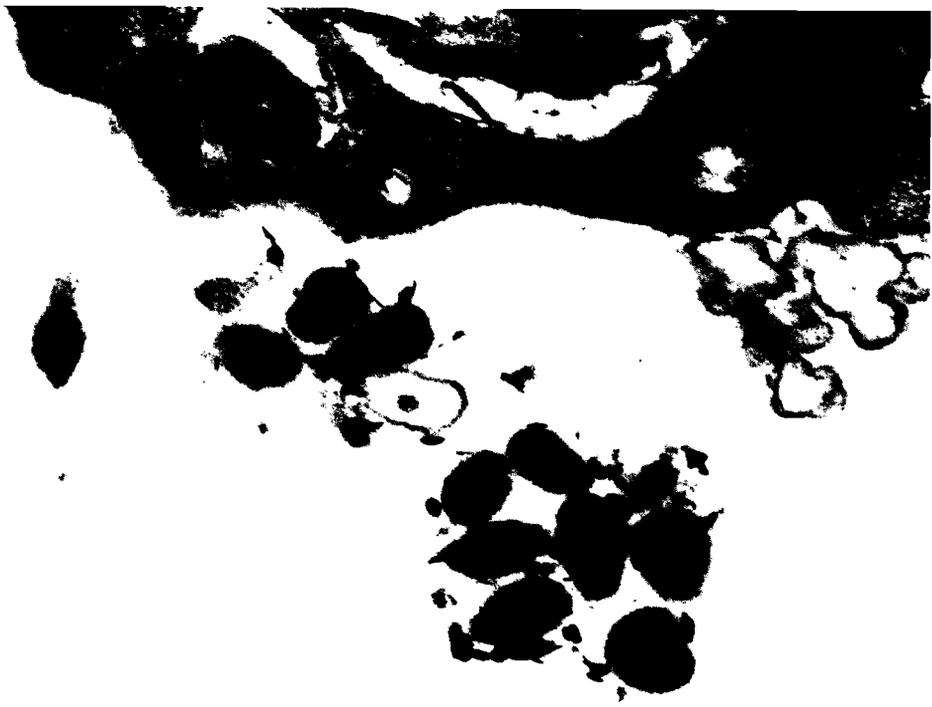


Figure 17. Electron micrograph of infected mouse peritoneal macrophage after 30 min. Phagocytosis of a viral particle is seen. (X 24,000) Uranyl acetate/lead citrate stain.



Figure 18. Electron micrograph of infected rabbit peritoneal macrophage after 30 min. Viral particles are seen both outside and inside the cell. (X 28,000) Uranyl acetate/lead citrate.



Figure 19. Electron micrograph of infected mouse peritoneal macrophage after 60 min. (X 8,000) Uranyl acetate/lead citrate.



Figure 20. Electron micrograph of infected rabbit peritoneal macrophage after 60 min. (X 24,000) Uranyl acetate/lead citrate.



Figure 21. Electron micrograph of infected mouse peritoneal macrophages after 90 min. (X 68,000) Uranyl acetate/lead citrate.



Figure 22. Electron micrograph of infected rabbit peritoneal macrophages after 90 min. (X 24,000) Uranyl acetate/lead citrate.



Figure 23. Electron micrograph of infected mouse peritoneal macrophages after 120 min. Viral core and its spikes are seen in the cytoplasm of the cell. (X 68,000) Uranyl acetate/lead citrate.





of purified  $^3\text{H}$ -thymidine labelled virus and the measurement of the rate at which viral DNA became accessible to DNase. Samples obtained from each experimental animal was divided into two distinct fractions, the acid soluble supernatant which contained broken down radioactive viral DNA, and an acid insoluble pellet which contained both viral cores and/or mature parental virus.

There was a lag of about 4 h in both mouse and rabbit macrophages before DNA became susceptible to the enzyme. This sensitization to DNase continued for about 4 h after which no more DNA became accessible (Table 1, 2). In contrast, very little radioactive material became acid soluble when samples were incubated at 37 C without DNase. Therefore, it was concluded that second stage uncoating of vaccinia occurs in both rabbit and mouse peritoneal macrophages.

#### Autoradiography of Infected Macrophages

As indicated earlier, both the normal mouse and rabbit peritoneal macrophages allowed first and second stage uncoating of the viral particle. After the early phases of infection it was unclear at what point in the replicative cycle there was a block which led to suppression of viral replication in mouse macrophages. Therefore, it was of interest to use autoradiography to determine whether DNA synthesis occurred in mouse macrophages or whether the DNA from the virus was degraded after being released from the core.

The amount of cytoplasmic silver-grain labelling, which

Table 1: Second stage uncoating assay of infected rabbit macrophages up to 8 h. Four h and 8 h samples show presence of broken down viral nucleotides in the supernatant of DNase treated lysates.

Table 2. Second stage uncoating assay of infected mouse macrophages up to 8 h. Both 4 h and 8 h samples treated with DNase show broken down tagged viral nucleotides in the supernatant.

TABLE 1  
RABBIT MACROPHAGES

time	LYSATE + TCA			LYSATE + TCA + DNase I		
	supernatant	pellet	total	supernatant	pellet	total
30 min	1.8%	82.9%	84.7%	3.1%	81.7%	84.8%
4 hrs	2.1%	84.6%	86.7%	78.9%	9.8%	88.7%
8 hrs	1.8%	91.8%	93.6%	80.1%	4.6%	84.7%

TABLE 2  
MOUSE MACROPHAGES

time	LYSATE + TCA			LYSATE + TCA + DNase I		
	supernatant	pellet	total	supernatant	pellet	total
30 min	2.6%	74.1%	76.7%	6.8%	73.8%	80.6%
4 hrs	1.9%	68.9%	70.8%	81.6%	12.8%	94.4%

was indicative of viral DNA synthesis, was observed to be much less in mouse cells than in rabbit cells (Fig. 25, 26, 27, 28, 29). Data obtained from 7,000 cell counts in each infected and control system indicated that there was a total increase in DNA synthesis in rabbit as opposed to mouse macrophages (Fig. 30). This was determined to be a statistically significant increase in the number of rabbit cells with viral induced sites of DNA synthesis in the cytoplasm (t test,  $p = 0.05$ ). Therefore, it was concluded that DNA synthesis was suppressed in normal mouse macrophages compared to rabbit peritoneal macrophages.

Figure 25. Autoradiograph of uninfected rabbit peritoneal macrophages after 8 h (X 400).

Figure 26. Autoradiograph of infected rabbit peritoneal macrophages at MOI of 1.00 after 8 h. Cytoplasmic silver grains are seen (X 400).



Figure 27. Autoradiograph of infected rabbit peritoneal macrophages at MOI of 1.00 after 8 h. Cytoplasmic silver grains are seen (X 400).

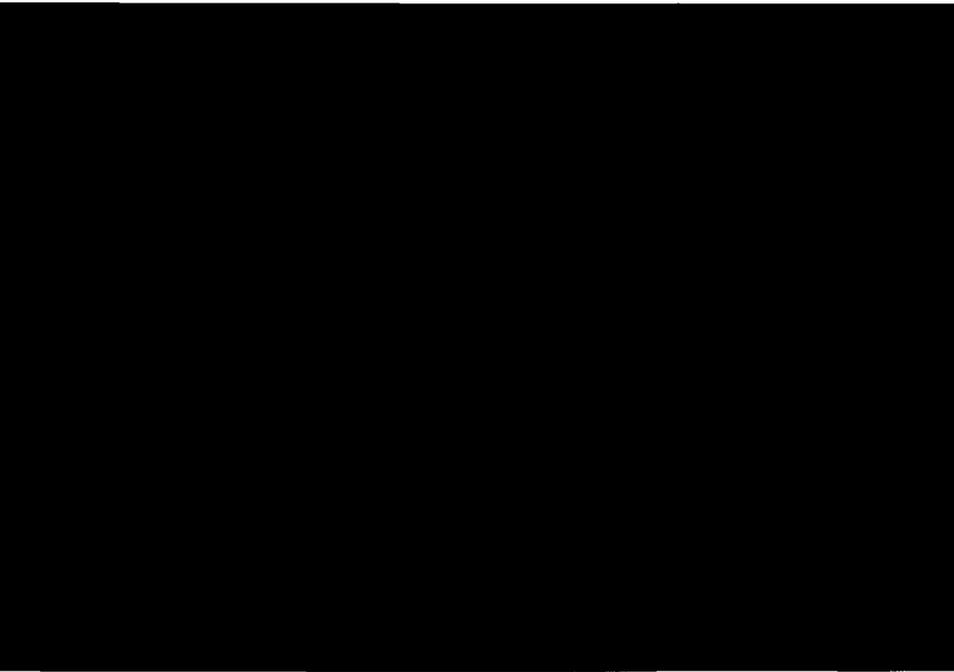


Figure 28. Autoradiograph of uninfected normal mouse peritoneal macrophages after 8 h (X 400).

Figure 29. Autoradiograph of infected normal mouse peritoneal macrophages after 8 h (X 400).

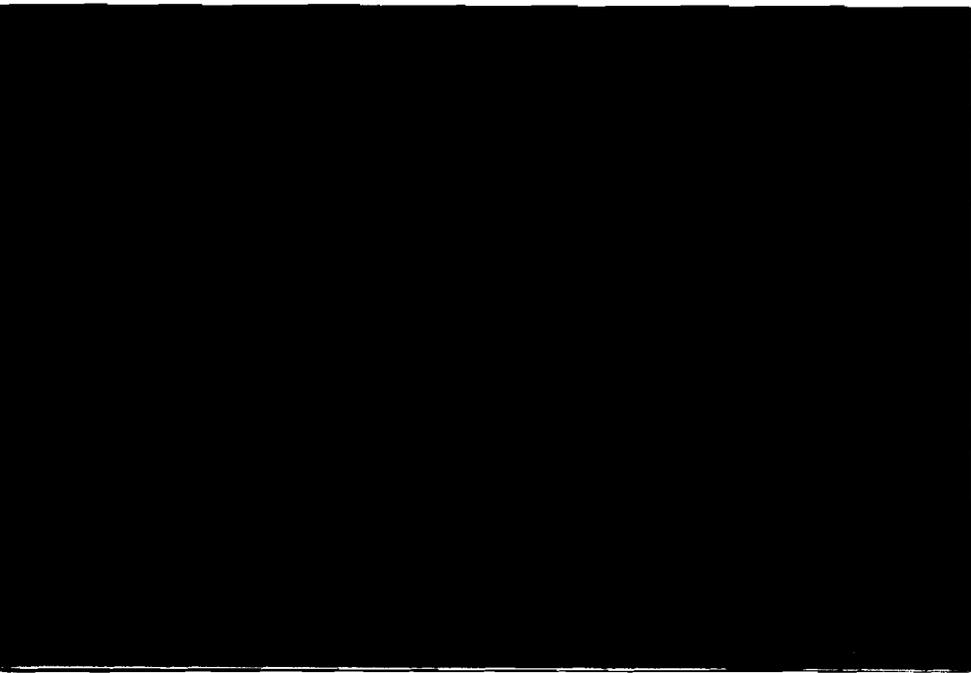
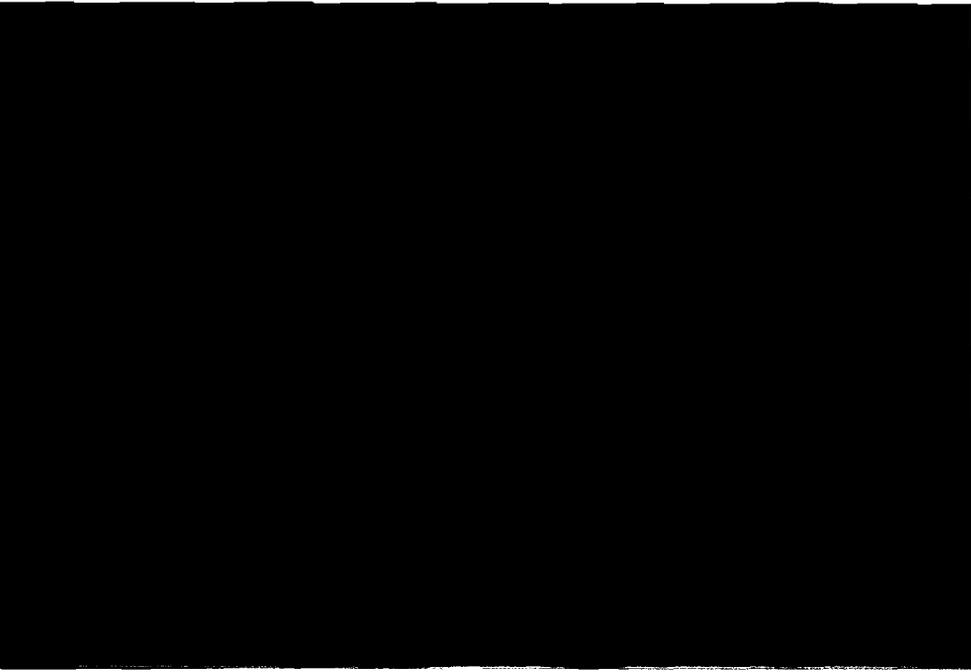
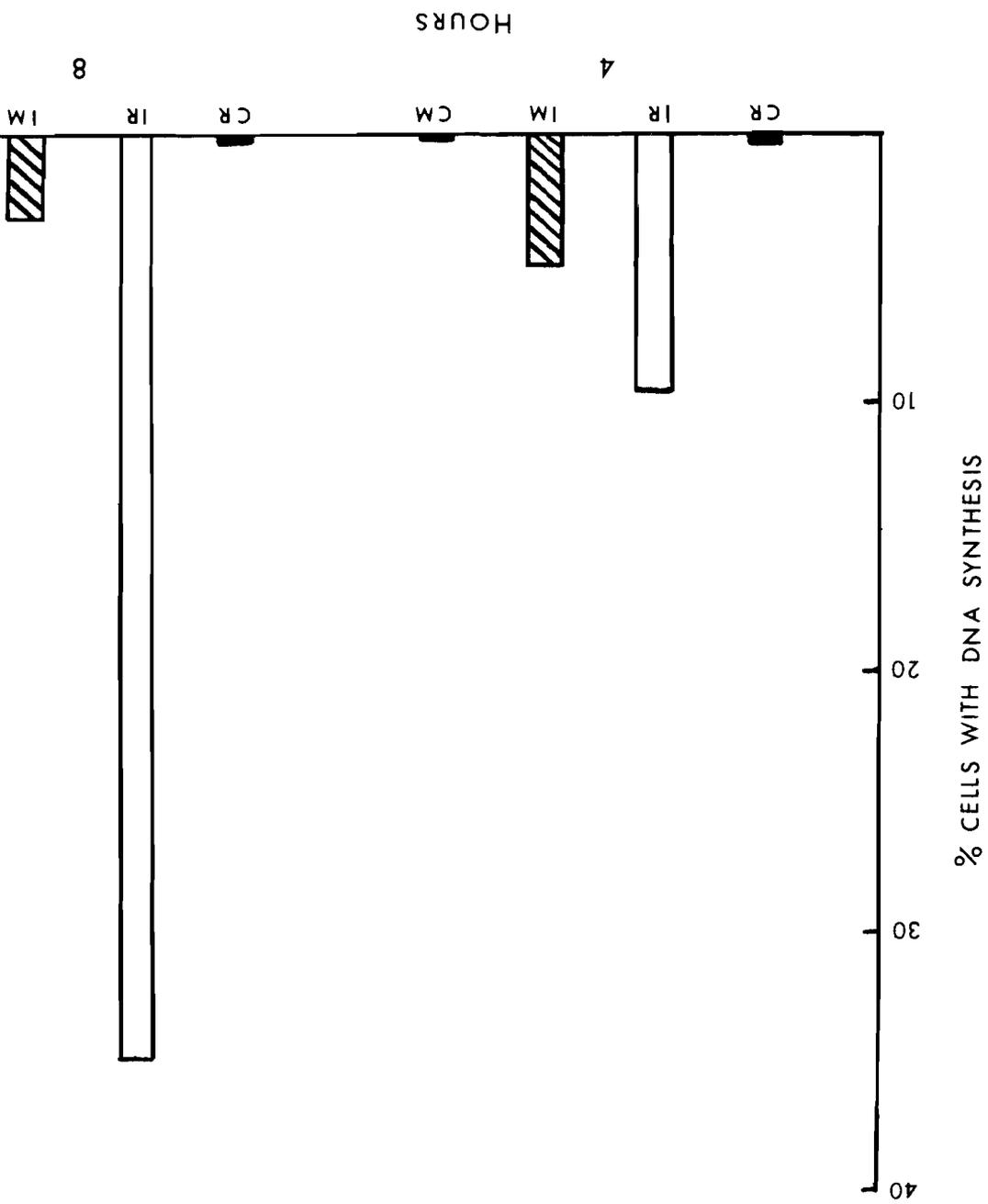


Figure 30. Comparison of viral induced DNA synthesis in normal mouse and rabbit peritoneal macrophages infected with IHD vaccinia up to 8 h.

CR = control rabbit (uninfected)  
IR = infected rabbit  
IM = infected mouse  
CM = control mouse (uninfected)



## DISCUSSION

The importance of the macrophage in combating viral infections is prominent at several levels. The macrophage plays a crucial role in inflammation and in the generation of specific cell-mediated and humoral immunity. However, many viruses replicate so quickly that appearance of cell-mediated and humoral immunity at two days post-infection is ineffective in clearing the heavy virus load. The fact that macrophages are capable of removing virus particles by phagocytosis provides an accessory mechanism for preventing virus spread to new host targets during that very critical period between the initiation of infection and the development of an effective immune response.

The encounter of an invading virus with a macrophage may determine whether the host supports or aborts a systemic viral infection. There are intraspecies genetic variations which are reflected in the macrophage system of the host which may account for innate or acquired immunity in virus infection. One good example of this is the resistance of normal mouse and the susceptibility of non-immune rabbit peritoneal macrophages to vaccinia virus.

The naturally occurring resistance of mouse macrophages to vaccinia infection was first reported by Nishmi and Bernkopf (1958). These workers found that mouse peritoneal macrophages, when infected in vitro with vaccinia, failed to support viral replication.

Greer (1973), using normal rabbit peritoneal macrophages, established vaccinia replication in these cells

while Tompkins et al (1970) showed that peritoneal macrophages from rabbits immunized with vaccinia would not support viral replication. Later, Buchemeier et al (1979) established that vaccinia replication is aborted during the condensation stage late in the viral replicative cycle within the immune rabbit macrophages.

The initial step in viral infection leading to viral progeny or death depending on the cell type involves particle penetration. Although there is disagreement concerning the mechanism(s) by which the viral core containing the viral genome gains access to the cytoplasmic matrix, attention has been directed toward two major pathways: 1) phagocytosis of viral particles with subsequent release of intact core from the phagocytic vacuole aided by lysosomal enzymes, and 2) fusion of cellular and viral membranes at the cell surface.

Dales (1963) concluded that the host cells contain enzymatic machinery necessary for carrying out the first stage of uncoating after virus has been phagocytized. Joklik (1966), using Hela cells as the host system, observed that in the process of first stage uncoating all the phospholipid of virus particles was converted to acid soluble substances, mainly glycerophosphate. Both of these authors assumed an enzymatic degradation of the outer membrane of vaccinia and subsequent release of the core into the host cytoplasm.

Silverstein et al (1973) proposed a different

mechanism of vaccinia penetration. He found that vaccinia virus enters its host cell within a phagocytic vacuole. The outer lipoprotein coat of the virus then interacts with the vacuolar membrane resulting in the lysis of the phagocytic vacuole and release of the DNA-containing viral core into the cytoplasmic matrix. This was supported by the fact that vaccinia virions, when neutralized with antibodies or denatured by heat treatment, were sequestered in the lysosomes of the host cell where they were completely degraded by hydrolytic enzymes. In contrast, when the untreated virus entered the cell, the viral core was released into the cytoplasmic matrix and the cell was destroyed. Chang and Metz (1976) used immunoferritin tagging and found that vaccinia fused with the L-cell membrane and antigenic components of the vaccinia virus envelope were rapidly and widely dispersed within the plane of the membrane, thereby supporting the work of Silverstein et al (1973) by confirming fusion of cellular and viral membrane at the cell surface.

Sodium suramin was used as a host lysosomal stabilizer to determine the activity of enzymes in first stage uncoating of vaccinia in mouse peritoneal macrophages. Results indicated the existence of infectious viral particles within mouse cells throughout 120 h as seen in figure 11. Although there was no increase in infectious viral titer, a histochemical examination of these cells revealed no change in the level of acid phosphatase particulate staining. Hence, it was concluded that first

stage uncoating of virus was not achieved due to lack of phagosome-lysosome fusion. This suggested that acid phosphatase enzymes of lysosomes were needed for first stage uncoating of the virus in mouse peritoneal macrophages. This is in agreement with findings of Joklik (1966) and Dales (1963), since they observed similar results when using Hela cells. However, further investigations concerning first stage uncoating of vaccinia are needed to establish the route by which viral core gains access to the macrophage cytoplasm.

Additional studies involving electron microscopy were undertaken to visually monitor the fate of the virus. Initially, cells were allowed to adsorb virions at 4 C for 90 min. Subsequent synchronized penetration was achieved by warming the cultures to 37 C. Results showed that the rate of phagocytosis and first stage uncoating up to 120 min was comparable in mouse and rabbit macrophages (Fig. 15, 16, 23, 24). Cells from both animals were equally efficient at phagocytizing and uncoating viral particles as evidenced by the appearance of viral cores in the cytoplasm of the mouse and rabbit macrophages. This observation established that both mouse and rabbit peritoneal macrophages allow first stage uncoating of vaccinia virus.

Studies concerning the second stage of uncoating were undertaken since 120 min electron micrograph observations established the occurrence of first stage uncoating as indicated by the presence of viral cores in both resistant

and susceptible cell cytoplasm. The usefulness of highly purified virus labelled with a radioactive tracer depended on a precise knowledge of the position of the tracer. Therefore, the status of viral DNA could be observed by testing whether label was specifically solubilized by the action of DNase, and for this purpose 3-H thymidine labelled virus was useful.

Second stage uncoating results revealed that when lysate samples were treated with DNase, the viral genome was degraded in both mouse and rabbit peritoneal macrophages. This was evident by the presence of radioactive material in the supernatant of infected cells. There was a 4 h lag before a significant count was observed in both susceptible and resistant macrophages. This sensitivity to viral DNA was observed up to 8 h. However, the supernatant of control samples showed consistently low amounts of radioactive material in the absence of DNase (Table 1, 2). These observations established the presence of second stage of uncoating in both resistant and susceptible cells.

Because both cell types were found to allow second stage uncoating, autoradiography was used to determine if additional steps of viral replication were occurring. Autoradiography observations of uninfected cells indicated consistently low (less than 0.8%) nuclear labelling throughout the 8 h period. This was to be expected because peritoneal macrophages are not known to divide in culture unless exogenous growth factors are added (Zeijst et al, 1978). When experiments were carried out through 8 h, an

increase in the number of cells showing cytoplasmic labelling was observed in infected rabbit macrophages (total of 36%). However, there was never a significant increase of cytoplasmic labelling in infected mouse peritoneal macrophages (total of 2.5%). This was due to the ability of mouse cells to inhibit viral DNA synthesis. The small amount of cytoplasmic labelling which was observed in mouse cell cultures was determined to be DNA synthesis in cells other than macrophages.

Evidence obtained points to the suppression of viral DNA synthesis in normal mouse peritoneal macrophages as a means of expressing innate immunity against vaccinia virus. The precise mechanism by which DNA synthesis is suppressed is not known. One of the possibilities is the presence of interferon in mouse cells. Bodo et al (1979) found that interferon acts by selectively inhibiting vaccinia-specific translation. Lewine et al (1967) went on to describe the mechanism of interferon action on cells infected with vaccinia and concluded that in interferon treated cells early viral ribonucleic acid is synthesized but no early virus protein or viral DNA is made. Pretreatment of cells with interferon also inhibited the complete uncoating of vaccinia virus. Thus, it is possible that the block early in the replicative cycle of vaccinia virus in mouse macrophages is due to the action of interferon. Interferon can be assayed by determining its effects on plaque formation by vesicular stomatitis virus (VSV), which is very

sensitive to interferon. Undoubtedly, future research will uncover additional macrophage-vaccinia interactions and biochemical roles for this fascinating host-parasite relation.

## SUMMARY

1. Results from virus growth assays indicated that vaccinia virus was incapable of multiplying in normal mouse peritoneal macrophages.
2. Studies concerning first stage uncoating of virus using sodium suramin as a host lysosome stabilizer indicated no change in viral titers throughout 120 h. Suramin treated cells also revealed no change in the level of acid phosphatase particulate staining. Hence, it was concluded that first stage uncoating of virus was not achieved due to lack of phagosome-lysosome fusion.
3. Electron micrograph studies revealed the presence of cytoplasmic viral cores in both susceptible and resistant cells. This established first stage uncoating of vaccinia in both rabbit and normal mouse peritoneal macrophages.
4. Second stage uncoating assays indicated the presence of degraded radioactive nucleotides in the supernatant of both mouse and rabbit cells. This established the second stage uncoating of vaccinia.
5. Autoradiography studies of infected mouse and rabbit macrophages showed that no significant viral DNA synthesis occurred in mouse cells when compared to rabbit cells. This indicated that the block of virus replicative cycle in mouse cells occurs during the first

half of the viral replicative cycle between the second stage uncoating and viral induced DNA synthesis.

6. The evidence of this study suggests that the suppression of viral DNA synthesis in normal mouse macrophages is a means of expressing innate immunity against vaccinia virus. One possible mechanism for the block early in the replicative cycle of vaccinia is the presence of interferon in mouse cells.

## LITERATURE CITED

## LITERATURE CITED

- Axline, S.G. 1970. Functional biochemistry of the macrophage. *Seminars in Hematology*. 7:147.
- Bodo, G., W. Scheirer, M. Suh, B. Schultze, I. Horak and C. Jungwirth. 1972. Protein synthesis in pox infected cells treated with interferon. *Virology*. 50:140-147.
- Buchmeier, N.A., R.G. Sydney, A.M. Frederick and W.E. Rawls. 1979. Abortive replication of vaccinia virus in activated rabbit macrophages. *Infection and Immunity*. 26:328-338.
- Chang, A. and D.H. Metz. 1976. Further investigation on the mode of entry of vaccinia virus into cells. *J. Gen. Virol.* 32:275-282.
- Clark, V.E. 1984. Mechanisms of genetically endowed macrophage resistance. Unpubl. Masters Thesis, Emporia State University. 55p.
- Cohn, Z.A. and E. Wiener. 1963. The particulate hydrolases of macrophages. I. Comparative enzymology, isolation, and properties. *J. Exp. Med.* 118:991.
- 
- \_\_\_\_\_. 1963. The particulate hydrolases of macrophages. II. Biochemical and morphological response to particle ingestion. *J. Exp. Med.* 118:1009.
- Dales, S. 1963. The uptake and development of vaccinia virus in strain L cells followed with labelled viral deoxyribonucleic acid. *The Journal of Cell Biology*. 18:51-72.
- Dales, S. and E.H. Mosbach. 1968. Vaccinia as a model for membrane biogenesis. *Virology*. 35:564-583.
- Easterbrook, K.B. 1966. Controlled degradation of vaccinia virions in vitro: an electron microscopic study. *J. Ultrastructure Research*. 14:484-496.
- Greer, B. 1973. Factors affecting uptake and replication of vaccinia virus in peritoneal macrophages in vitro. Unpubl. Masters Thesis, Emporia State University. 61p.
- Hayat, M.A. 1970. Principles and techniques of electron microscopy. Van Nostrand Reinhold Company. 31-34. New York, New York.

- Hood, G. 1978. Immunology. The Benjamin/Cummings Publishing Company, Inc. 63-65.
- Joklik, W.K. 1964. The intracellular fate of rabbit pox virus rendered non-infectious by various reagents. *Virology*. 22:620.
- \_\_\_\_\_. 1964. The intracellular uncoating of pox virus DNA. I. The fate of radioactively labelled rabbit pox virus. *J. Mol. Bio.* 8:263-276.
- \_\_\_\_\_. 1966. The pox viruses. *Bact. Rev.* 30:33-60.
- Lewine, S.W., E. Magee, R. Hamilton and O.V. Miller. 1976. Effect of interferon on early enzyme and viral DNA synthesis in vaccinia virus infection. *Virology*. 32:33-40.
- Metchnikoff, E. 1892. LeCons sur la pathologie comparee de l'inflammation. Cited in *Mononuclear Phagocytes*. (R. Van Furth, ed.) 1-6. Blackwell Scientific Publications, Oxford.
- Nishmi, M. and H. Bernkoff. 1958. Toxic effect of vaccinia virus on leukocytes in vitro. *J. Immunol.* 81:460-466.
- Schnyder, J. and M. Baggiolini. 1978. Secretion of lysosomal hydrolases by stimulated and non stimulated macrophages. *J. Exp. Med.* 148:435-450.
- Schultz, W.W. 1966. An in vitro study of the susceptibility of peritoneal macrophages from mice and rabbits to vaccinia virus. Unpubl. Masters Thesis, Emporia State University. 81p.
- Shidani, B. 1980. A study of factors which affect the resistance of alveolar macrophages to vaccinia virus. Unpubl. Masters Thesis, Emporia State University. 12p.
- Silverstein, S.C. 1970. Macrophages and viral immunity. *Seminars in Hematology*. 7:185-214.
- Silverstein, S.C., C. Astell and D. Levin. 1973. The role of lysosomes in the uncoating and activation of the reovirus genome. *Advances in the Biosciences*. 11:3-26.
- Stuart, A.E. and J.A. Habeshaw. 1973. Phagocytes in vitro. Cited in *Handbook of Experimental Immunology*. (second edition). 24.1-24.26. Blackwell Scientific Publication, Oxford.
- Tompkins, W.A.F., J.M. Zarling and W.E. Rauls. 1970. In

vitro assessment of cellular immunity to vaccinia virus: contribution of lymphocytes and macrophages. Infect. and Immun. 2:783.

Tsan, M.F. and R.D. Berlin. 1971. J. Exp. Med. 134: 1016-1035.

Ward, J.E. 1980. The interaction of stimulated mouse macrophages with vaccinia virus in vitro. Unpubl. Masters Thesis, Emporia State University. 50p.

Zeijst, V., B.A.M. Stewart. 1978. Proliferative capacity of mouse peritoneal macrophages in vitro. J. Exp. Med. 1253.