AN ABSTRACT OF THE THESIS OF

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in Biology presented on December 20, 1991

Title: The Study of DNA Polymerase in Resistant and Susceptible Macrophages Infected with Vaccinia Virus

Abstract approved: Helen McGhee PhD

Studies have demonstrated that mouse peritoneal macrophages restrict vaccinia virus replication in vitro whereas rabbit peritoneal macrophages do not. Because this in vitro pattern parrallels the resistance and susceptibility to vaccinia infection by the respective macrophage donors, it is reasonable to assume that information concerning the intracellular mechanisms which account for these differences would be valuable to the understanding of innate host resistance.

Previous studies revealed that although there is no difference in the phagocytosis, first stage uncoating, and the second stage uncoating of vaccinia in mouse and rabbit macrophages, there was a difference in viral induced DNA synthesis. DNA synthesis occurred in rabbit macrophages but was not found in mouse macrophages (Ward, 1980; Milligan, 1983; Kashanchi, 1986). These findings indicated that the inhibition of vaccinia by mouse macrophages took place after second stage uncoating but before viral DNA synthesis.

Mpwo (1989) investigated the question of whether vaccinia virus induced protein synthesis occurred. He used as a marker of early protein synthesis the enzyme, thymidine kinase. His studies revealed no thymidine phosphorylating activity in mouse macrophages. The present study was undertaken in order to extend the findings of

intracellular mechanisms involved in the innate resistance of mouse macrophages to vaccinia virus.

It was important to determine if another early enzyme, DNA polymerase was made. This enzyme is directly involved with DNA synthesis. Biochemical studies revealed no DNA polymerase activity in mouse macrophages in comparison to rabbit macrophages and mouse fibroblasts.

Studies concerning the second stage uncoating revealed no occurrence of second stage uncoating in the mouse macrophages treated with the protein synthesis inhibitor Streptovitacin A. This study indicated that the host must synthesize protein at the time of infection for the release of viral DNA. This evidence suggests that the enzyme responsible for second stage uncoating is being synthesized in the mouse macrophages and the second stage uncoating is viral induced. The two early enzymes responsible for DNA synthesis are not produced. Therefore, mouse peritoneal macrophages express innate resistance against vaccinia by suppressing the synthesis of early enzymes thymidine kinase and DNA polymerase which leads to suppression of DNA synthesis.

THE STUDY OF DNA POLYMERASE IN RESISTANT AND SUSCEPTIBLE MACROPHAGES INFECTED WITH VACCINIA VIRUS

A Thesis

Presented to

the Division of Biological Sciences

Emporia State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

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December 1991

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ACKNOWLEDGMENTS

I wish to thank my committee members, Dr. Rodney Sobieski and Dr. Alfred Ericson for their time and patience. I would also like to thank Dr. Fatah Kashanchi for his assistance and Roger Ferguson for providing animals needed. I would like to express my warmest thanks to my family members for their support, both emotional and financial. Finally, I wish to express my deepest appreciation to Dr. Helen McElree for her encouragement and guidance throughout my study.

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INTRODUCTION

One of the most important roles of the immune system is host defense against infection. There are two major kinds of mechanisms involved in host defense. One is innate resistance and the other is acquired immunity. Innate resistance is due to inborn species characteristics. It is developed through inheritance and normally passed on to new generations. Acquired immunity develops in an individual as a result of physiological responses to an infection or immunization and is responsible for long term specific protection against an infection.

Today, a great deal of information is available about the role of macrophages in acquired immunity. On the other hand, relatively little is known about the mechanisms of innate immunity. However, there is evidence that macrophages play an important role in innate immunity (Nishmi and Niecikowski, 1963; Schultz, 1966; Milligan, 1983; Clark, 1984; Kashanchi, 1986).

Macrophages, which are widely dispersed in the body, are derived from circulating monocytes (Hood, 1978). The macrophages, and their precursors that are distributed throughout the body, are referred to as the "mononuclear-phagocyte system" (Johnston, 1988). Cells comprising the mononuclear phagocytic system 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).

The cytology and physiology of macrophages vary depending on

of specific immunity and by serving as "accessory" cells to lymphocytes by releasing soluble factors. Macrophages also play an important role in removing dead or damaged cells and repairing wounds (Rabinovitch, 1968, 1970).

There is a good evidence for a protective role of macrophages in viral infections. Johnson (1964), in his study of age dependent susceptibility of mice to herpes simplex virus (HSV), showed that although the uptake of virus by adult and newborn mouse macrophages was similar, the newborn mouse macrophages were permissive for infection whereas adult mouse macrophages were not.

Hirsch et al. (1970) showed that transplanting stimulated adult macrophages into syngenic suckling mice protected the latter from infection with HSV. Also, they mentioned that mature macrophages were able to phagocytize and destroy the virus more efficiently than the immature macrophages

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.

Replication of poxviruses in macrophages is related to both virus and cell strain (Silverstein, 1970). Vaccinia virus replicates in normal rabbit macrophages (Beard et al, 1938; Florman et al., 1942; Greer, 1973), but not in normal mouse macrophages (Nishmi and Bernkopf, 1958; Nishmi and Niecikowski, 1963; Glasglow, 1965; Schultz, 1966; Ward, 1980; Kashanchi, 1986).

Vaccinia virus is a member of the poxvirus group. It is a

brick-shaped virus approximately 3000 A x 2000 A x 2000 A (Dales, 1965; Joklik, 1968). It has a lipoprotein envelope (Dales, 1968) which encloses a biconcave protein core containing the viral genome. Five to 10 % of its genome is made up of single-stranded DNA with the rest being double-stranded. Vaccinia virus is composed of 5 % DNA, 2 % lipid, 2 % phospholipid and the rest is protein (Joklik, 1966). Two lateral bodies lie next to the viral core. The function of these lateral bodies is unknown.

The genome of vaccinia virus encodes information for about 230 different polypeptides of which approximately 100 are incorporated into the mature viron (Moss, 1985). Acrylamide gel analysis indicates that there are at least 17 protein species (Holowezak and Joklik, 1967).

When the virus comes in contact with the cell surface of the macrophage, phagocytosis is initiated. The cell extends a pseudopod around the virus and incorporates the virus into phagocytic vacuoles called phagosomes. The phagosome then fuse with lysosomes which contain a variety of hydrolytic enzymes. The virus loses its first coat in an event termed first stage uncoating followed by release of the viral core into the cytoplasmic matrix (Dales, 1965; Joklik, 1968). The first stage uncoating takes place immediately after infection (Joklik, 1966). Following the first stage uncoating, viral induced early proteins are synthesized. Some of these early proteins include thymidine kinase, DNA polymerase and alkaline DNase (Joklik, 1968; McAuslan, 1966).

The second stage uncoating occurs only after a lag period and

results in the release of naked viral DNA from the viral core. The second stage of uncoating is characterized by its dependence on protein synthesis and the sensitization of vaccinia virus DNA to DNase (Joklik, 1963).

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).

Studies in this laboratory have concentrated on the vaccinia virus-macrophage model in an attempt to determine the mechanism that allows mouse macrophages to abort vaccinia infection. Schultz (1966) examined vaccinia cytopathic effects in both rabbit and mouse peritoneal macrophages. He used acid phosphatase stain and found greater activation of lysosomes in vaccinia infected mouse macrophages than in infected rabbit macrophages. Therefore, he concluded that the mechanism involved in the resistance of mouse macrophages and the susceptibility of rabbit macrophages were mainly due to the presence of hydrolytic enzymes in the mouse macrophages. Using autoradiography, Milligan (1983) showed that viral DNA synthesis did not occur in the non-permissive mouse cell but that there was viral DNA synthesis in the permissive rabbit cell. Ward (1980), using autoradiography, showed that viral DNA synthesis did not occur in the non-permissive mouse cell.

Silverstein (1970), using electron microscopy and biochemical experiments employing H^3-thymidine labeled IDH strain of vaccinia virus showed that first stage uncoating took place, but no second

stage uncoating.

Eventhough Silverstein reported that there was no second stage uncoating, Kashanchi (1986) showed evidence of both the first and second stages of uncoating. His electron micrograph studies revealed the presence of cytoplasmic viral cores in both susceptible and resistant cells. This indicated the first stage uncoating of vaccinia in both rabbit and mouse peritoneal macrophages. Kashanchi showed evidence for second stage uncoating of vaccinia in both susceptible and resistant cells by the presence of degraded radioactive DNA nucleotides in the supernatant of both mouse and rabbit cells. His autoradiography studies of infected mouse and rabbit macrophages revealed that no significant viral DNA synthesis occurred in mouse cells when compared to rabbit cells. He concluded that the block of the virus replicative cycle occurred somewhere between the second stage of uncoating and viral DNA synthesis.

Mpwo (1989) confirmed the work of Kashanchi by showing evidence for first and second stages of uncoating. Mpwo, using electron microscopy, showed evidence for first stage uncoating in both rabbit and mouse cells. His biochemical tests revealed that viral DNA was converted to DNase sensitive form in both the susceptible rabbit macrophages and the resistant mouse macrophages, thus confirming the occurrence of the second stage of uncoating. Because second stage uncoating requires the synthesis of a viral directed enzyme (Moss, 1985), it was important to determine if other early enzymes were being produced. Mpwo obtained results indicating that an early enzyme involved with DNA synthesis, thymidine kinase, was not

produced in mouse macrophages.

The present study was undertaken to examine the mechanisms which allow normal mouse peritoneal macrophages to suppress vaccinia replication. In particular, answers were sought to the following questions: Is DNA polymerase produced in the resistant mouse macrophages? A point of interest is the fact that thymidine kinase is synthesized by permissive cells before second stage uncoating and DNA polymerase is produced after second stage uncoating. This enzyme assay can serve as an important marker which can provide additional information regarding early biochemical events which occur or do not occur in the non-permissive mouse macrophages as compared to the permissive rabbit macrophage. Is the second stage uncoating in the resistant cell, viral induced or non-viral induced? According to Novikoff et al. (1964), the second stage uncoating in non-permissive mouse macrophages could be due to secretion of lysosomal enzyme by the host cell. Radioactively labelled virus and biochemical tests were used to answer the above questions.

MATERIALS AND METHODS

Experimental Animals

Young adult albino mice and adult New Zealand white rabbits of both sexes were used in the experimental procedures.

Cell Culture Media

All cells used in this study were cultured in Eagles Minimal Essential Medium (MEM) containing combiotic (Penicillin G, 200 units per ml, Streptomycin, 100 units per ml) and sterile inactivated fetal calf serum (FCS) at a final concentration of 10 % or 2 % as needed. MEM containing 2 units of heparin per ml was used for harvesting both mouse and rabbit macrophages. Sterile 0.5 % sodium bicarbonate was used to adjust the pH of the cultures when necessary.

A stock solution of trypsin (Sigma, S.P. = 12,400 BAEE units/mg protein) 5 % W/V was prepared in Dulbecco's phosphate buffer solution (DPBS) which contained no Mg^{+2} or Ca^{+2}. This preparation was membrane filtered, aliquated in 5 ml amounts, and stored at -20 C. The trypsinizing solution contained the final concentration of 0.25 % trypsin and 0.02 % EDTA.

Cell Culture Procedures

Mouse Peritoneal Macrophages

Macrophages were harvested by injecting 5 ml of heparinized MEM into the peritoneal cavity of mice followed by several minutes of massage of the abdominal wall to suspend the cells. After aseptically opening the peritoneal cavity, the cell suspension was

removed and counted. The cell count was adjusted to 4.0 X 10^6 cells per ml and planted in cell culture flasks (5 ml) and flying coverslip tubes (1 ml).

Cells were allowed to settle and attach to the container surface during 2 h of incubation at 37 C. The supernatant was removed and replaced with MEM supplemented with 10 % FCS. All cultures were incubated at 37 C.

Rabbit Peritoneal Macrophages

In order to elicit peritoneal macrophages in rabbits, 50 ml of sterile mineral oil was introduced intra peritoneally (IP). Three days later the rabbits were sacrificed by air embolism and the cell suspension collected from the peritoneal cavity in heparinized MEM. The cell suspension was placed into a separatory funnel and held 15 min at 4 C to allow the oil to separate. The aqueous phase, which contained the cells, was drained into a flask. The cell count was adjusted to 4 X 10^6 cells per ml, planted, and incubated as described for the mouse cell preparations.

Mouse Fibroblast Cultures

The pregnant mice were sacrificed by cervical dislocation between the tenth to sixteenth day of gestation. The abdomen was aseptically opened to expose the uterus, the embryos were removed, decapitated, and transferred to a petri dish containing sterile DPBS. The embryos were minced with a sterile scalpel into 1 mm^3 pieces and the embryo fragments were placed in a 100 ml of trypsinizing solution consisting of 95 ml DPBS, 5 ml of 0.25 % trypsin and 0.2 ml of 10 %

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 was added to the trypsinizing flask to stop the action of trypsin. The cells were filtered by pouring through a sterile cheese cloth filter to remove the cell debris. Then the cell suspension was washed twice with DPBS, centrified at 2000 rpm for 10 min, and the cell count adjusted to 3.0×10^6 cells per ml with MEM plus 10 % FCS. Five ml of cell suspension per flask was planted in 25 cm^2 cell culture flasks. Cell suspensions were incubated at 37 C until the cells monolayered and the cell monolayers were used for viral titration.

Virus Propagation

The IHD strain of virus, obtained from the American Type Culture Collection, was used in this study. Approximately 50 ml of mouse embryonic cell suspension was incubated at 37 C until the cells were monolayered. Then the medium was removed and the cell monolayer was washed twice with DPBS and inoculated with 10 plaque forming units (Pfu) of IHD vaccinia virus per ml in MEM containing 2 % FCS for a total of 10 ml. The cultures were incubated for 2 h at 37 C. During this period, flasks were gently agitated every 5 min to allow for adsorption to take place. At the end of adsorption, the inoculating medium was removed, the cells washed twice in situ with DPBS and replaced with 50 ml MEM containing 10 % FCS. The cells were incubated at 37 C until viral induced cell destruction was seen.

Radioactively labelled virus was prepared by infecting the mouse

embryo monolayers. After viral adsorption the inoculating medium was removed and washed twice with DPBS. Viral DNA was labelled by incubating the infected cultures in 50 ml of MEM containing H^3-thymidine (ICN Radiochemicals) at a final concentration of 5 uCI per ml. Cultures were incubated at 37 C until extensive destruction was seen.

Harvesting of Virus

Radioactively labelled virus was harvested by subjecting the infected cell cultures to 3 cycles of freeze-thaw to release intracellular virus. The cell debris was removed by centrifuging for 10 min at 2500 rpm. The viral containing supernatant was pooled, aliquted in 10 ml amounts, and stored at -20 C.

Virus Titration

Ten fold dilutions of radioactively labelled virus were prepared in MEM plus 2 % FCS. The dilutions were from 10^{-1} to 10^{-10}. Two ml of the 6 highest dilutions was inoculated into culture flasks of monolayered mouse embryo fibroblasts. Infected cell cultures were incubated at 37 C for 2 h. During this period, flasks were gently agitated every 5 min to allow adsorption to take place. The medium containing the unadsorbed virus was removed, washed twice with DPBS, and replaced with MEM and 10 % FCS. The flasks were incubated at 37 C until plaque formation was observed. The medium was removed and the monolayers were stained with Gram's crystal violet diluted 1:10 with water for approximately 3 min. The excess Gram's crystal violet was removed, the monolayers were washed with cold water, and the

plaques were counted. The final concentration of the virus was expressed as plaque forming units (pfu) per ml.

May Grunwald Giemsa Stain

Cells on coverslips were 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 5-10 min. The coverslips were mounted cell side down in Kleermount xylene solution on glass slides.

Assay for DNA-Polymerase

In order to determine if the early viral directed enzyme, DNA polymerase, is made in the resistant mouse macrophages, virus was adsorbed to macrophages (2:1 ratio) at 4 C for 30 min. Cells were infected with vaccinia virus at 4 C to allow viral attachment but not penetration into the cell (Joklik, 1963). The unadsorbed virus was removed and replaced with MEM plus 10 % FCS and the virus-cell complexes were incubated at 37 C, thereby initiating a synchronous entry of virus into the cell. At 3 h and 6 h after warming, the cells were removed from the culture flasks and centrifuged at 2500 rpm for 10 min to deposit the cells. The cell pellet was washed once with DPBS and resuspended in 300 ul of DPBS and .001 % Triton X-100 (Hymer and Kuff, 1964). Cells were placed in an ice bath and disrupted with a Dounce homogenizer (Penman et al., 1963). The disrupted cells were centrifuged for 10 min using a micro centrifuge and the supernatant, containing the cytoplasmic fractions, was collected as a source of the enzyme (Penman et al., 1963).

DNA polymerase activity was tested by using a reaction mixture consisting of: 5 ul of 10 X Nick-translation buffer (NTB), 5 ul of 10 mM dATP, dCTP, and dTTP, 5 ul of 1 mg/ml heated calf thymus DNA template, 0.5 ul of 1 mg/ml heated calf thymus DNA primer, 33.5 ul enzyme and 1 ul of 10 mCI per ml p^{32} ATP.

After incubation for 1 h at 37 C, the reaction mixture from each sample was applied onto separate 27 mm DE81 filter paper and air dried. Then the filter papers were soaked twice in 200 ml of 0.5 M Na2HP04 for 6 min. After soaking the filter papers in Na2HP04 buffer, they were rinsed in deionized water and then in 95 % ethanol. The filters papers were air dried and placed in scintillation vials. Five ml of scintillation cocktail (Scintiverse II) was added into each vial and DNA polymerase activity was assayed by determining the amount of ^{32}P-ATP incorporated into DNA using a LKB liquid scintillation counter. The positive control systems consisted of permissive mouse embryo fibroblasts and rabbit macrophages. The experimental system was the non-permissive mouse macrophages. Klenow, which is a fragment of E. coli DNA polymerase, served as an additional control.

Second Stage Uncoating Assay

H^3 DNA labelled virus was adsorbed to macrophages (10:1 ratio) at 4 C for 30 min which allowed for viral attachment but not penetration into the cell (Joklik, 1963). The unadsorbed virus was removed and the virus-cell complexes were incubated at 37 C to promote the synchronous entry of virus into the cells. At 30 min, 4 h, and 8 h

after incubation, the cells were scraped from the cell culture flasks and centrifuged at 2500 rpm for 10 min to deposit cells. The cell pellet was washed once with DPBS and processed by resuspending it in 2.2 ml of 0.01 M NaH_2PO_4 and 0.01 M $MgCl_2$ at pH7 (Kashanchi, 1986). Cells were broken with a Donce homogenizer using 40 strokes (Penman et al., 1963). It was followed by 2 cycles of freeze-thaw to release all viral DNA present from the cytoplasmic matrix of the cells (Buchmeier et al., 1979). A total radioactivity count was obtained at this point by taking 0.2 ml quantities from each sample and counting them on a LKB liquid scintillation counter (Kashanchi, 1986). One half of the remaining sample was treated with 0.2 ml of 50 % stock TCA and the other half with 0.2 ml of 50 % stock TCA plus 0.1 ml 500 mg/ml of DNase I (Buchmeier et al., 1979). Both halves were incubated at 37 C for 30 min. Then they were held overnight at 4 C and centrifuged at 2500 rpm for 15 min to separate supernatant from TCA precipitate. The percentage of radioactivity in each sample was calculated by dividing experimental counts over total count times 100. The positive control system consisted of infected mouse macrophage cultures and the experimental system consisted of infected mouse macrophage cultures treated with 10ug/ml of Streptovitacin A (Dales, 1965).

RESULTS

DNA Polymerase Activity

DNA polymerase activity was detected by determining the amount of ^{32}P-ATP incorporated into the enzyme product, DNA (Jungwirth and Joklik, 1965). The positive control systems consisted of infected and non-infected permissive mouse embryo fibroblasts and rabbit macrophages. The experimental system consisted of infected and non-infected mouse macrophages. Klenow, which is a fragment of E. coli DNA polymerase, served as an additional control.

Table 1 indicates the activity of DNA polymerase in mouse embryo fibroblasts at 3 and 6 h incubation at 37 C. The activity of DNA polymerase in uninfected mouse embryo fibroblasts at 6 h incubation was 4607.4 cpm, whereas in infected mouse embryo fibroblasts the activity was 590250.6 cpm (Table 1). The results, as expected, indicated that the DNA polymerase activity was significantly increased in infected mouse embryo fibroblasts after 6 h incubation at 37 C. However, there was no significant increase of DNA polymerase activity in infected mouse embryo fibroblasts at 3 h incubation (Table 1).

Table 2 presents the results when DNA polymerase activity in permissive mouse embryo fibroblasts were compared to the enzyme activity in non-permissive mouse macrophages. Vaccinia infected mouse fibroblasts showed a significant amount of DNA polymerase activity (879601.4 cpm) as compared to the uninfected control (183.6 cpm). In contrast, infected mouse macrophage cultures yielded a

Table 1. DNA polymerase activity in mouse fibroblasts at 3 and 6 h incubation at 37 C.

	MOUSE FIBROBLASTS	
	DNA POLYMERASE ACTIVITY (CPM)	
Hours	Control	Infected
3	5803.8	6409.4
6	4607.4	590250.6

Table 2. DNA polymerase activity in mouse fibroblasts and mouse macrophages at 6 h of incubation at 37 C.

	DNA POLYMERASE ACTIVITY (CPM)
Mouse Fibroblasts	183.6
Infected Mouse Fibroblasts	879601.4
Mouse Macrophages	932.0
Infected Mouse Macrophages	1566.4
Klenow	716454.5

count of 1566.4 cpm which was only slightly more than the uninfected mouse macrophages count of 932.0 cpm. Therefore, based on these data it appears that mouse macrophages as compared to mouse fibroblasts do not synthesize DNA polymerase as a result of vaccinia infection.

Because, the data presented in Table 2 were based on a control system of cells which were fibroblasts rather than macrophages, a second set of experiments were done in which rabbit macrophages were used as the permissive host cells. The findings presented in Table 3, clearly show that infected rabbit macrophages exhibit enzyme activity (2991.8 cpm) as compared to infected mouse macrophages which did not (317.1 cpm).

Therefore, it appears that both mouse fibroblasts and rabbit macrophages, which are permissive for the vaccinia virus infection, produced the early enzyme DNA polymerase but the non-permissive mouse cell did not.

Assay for Second Stage Uncoating

It was important to determine whether the second stage uncoating in the non-permissive mouse macrophage was viral induced or non-viral induced. The second stage uncoating was assayed by monitoring the amount of ^{3}H-Thymidine labelled viral DNA which became accessible to DNase. The positive control system consisted of infected non-permissive mouse macrophage cultures and the experimental system consisted of infected non-permissive mouse macrophage cultures treated with the protein synthesis inhibitor Streptovitacin A (Dales, 1965).

Table 3. DNA polymerase activity in mouse macrophages and rabbit macrophages at 6 h incubation at 37 C.

	DNA POLYMERASE ACTIVITY (CPM)
Mouse Macrophages	298.0
Infected Mouse Macrophages	317.1
Rabbit Macrophages	302.4
Infected Rabbit Macrophages	2991.8
Klenow	1806.5

Table 4 presents the results of second stage uncoating in untreated infected non-permissive mouse macrophage cultures. At 30 min post infection, the acid soluble supernatant contained little radioactivity, indicating that at this time there was no sensitization of DNA to DNase and, therefore, no second stage of uncoating (Table 4). At 4 and 8 h post infection in untreated mouse macrophages, the viral DNA became DNase sensitive, as evidenced by the higher radioactivity count in the supernatant as compared to the precipitate (Table 4). At 4 h post infection in untreated mouse macrophages, the radioactivity count in the supernatant was 52.7 % and in the pellet was 46.1 %. At 8 h post infection in untreated mouse macrophages, the radioactivity count in the supernatant was 73.2 % and in the pellet was 26.8 % (Table 4). The findings presented in Table 4 indicate the occurrence of second stage uncoating in untreated mouse macrophages. In contrast, little radioactive material was acid soluble when the samples were incubated without DNase in the infected mouse macrophages.

Table 5 presents the results of second stage uncoating assay of infected mouse macrophages treated with the protein synthesis inhibitor Streptovitacin A. The viral DNA did not become DNase sensitive, as evidenced by a lower radioactivity count in the supernatant than in the precipitate (Table 5). At 4 h post infection in Streptovitacin A treated mouse macrophages, the radioactivity count in the supernatant was 35.3 % and in the pellet was 64.1 % and at 8 h post infection in Streptovitacin A treated mouse macrophages,

Table 4. Second stage uncoating assay of infected mouse macrophages up to 8 h.

	MOUSE MACROPHAGES					
	LYSATE + TCA			LYSATE + TCA + DNase I		
Time	Supernatant	Pellet	Total	Supernatant	Pellet	Total
30 min.	12.8 %	56.9 %	69.7 %	14.7 %	79.6 %	94.3 %
4 hrs	29.3 %	56.3 %	85.6 %	52.7 %	46.1 %	98.8 %
8 hrs	33.8 %	49.2 %	83.0 %	73.2 %	26.8 %	100.0 %

Table 5. Second stage uncoating assay of infected mouse macrophages treated with protein synthesis inhibitor streptovitacin A up to 8 h.

MOUSE MACROPHAGES TREATED WITH STREPTOVITACIN A						
	LYSATE + TCA			LYSATE + TCA + DNase I		
Time	Supernatant	Pellet	Total	Supernatant	Pellet	Total
30 min.	17.6 %	50.8 %	68.4 %	38.1 %	60.6 %	98.7 %
4 hrs	9.2 %	55.6 %	64.8 %	35.3 %	64.1 %	99.4 %
8 hrs	23.1 %	52.2 %	75.3 %	39.9 %	59.4 %	99.3 %

the radioactivity count in the supernatant was 39.9 % and in the pellet was 59.4 % (Table 5). The findings, presented in Table 5, clearly indicate that the second stage uncoating did not occur when protein synthesis was inhibited by treating the mouse macrophages with Streptovitacin A.

DISCUSSION

Macrohages play an important role in host resistance or susceptibility to viral infection. Previous studies have demonstrated that mouse peritoneal macrophages restrict vaccinia virus replication in vitro whereas rabbit peritoneal macrophages do not (Nishmi and Bernkopf, 1958; Schultz, 1966; Greer, 1973; Ward, 1980; Kashanchi, 1986; Mpwo, 1989). Because this in vitro pattern parallels the resistance and susceptibility to vaccinia infection by the respective macrophage donors, it is reasonable to assume that information concerning the intracellular mechanisms which account for these differences would be valuable to the understanding of innate host resistance.

In earlier studies it was found that although there is no difference in the phagocytosis, first stage uncoating, and second stage uncoating of vaccinia in mouse and rabbit macrophages (Kashanchi, 1986; Mpwo, 1989), there was a difference in viral induced DNA synthesis. DNA synthesis occurred in the permissive rabbit macrophages but was not found in the non-permissive mouse macrophages (Ward, 1980; Milligan, 1983; Kashanchi, 1986). These findings were interpreted to mean that the inhibition of vaccinia by mouse macrophages took place after second stage uncoating but before viral DNA synthesis (Kashanchi, 1986).

In a follow up study Mpwo (1989) investigated the question of whether vaccinia virus induced protein synthesis occurred. He used as a marker of early protein synthesis the enzyme, thymidine kinase (McAuslin, 1963; Esteban and Metz, 1972). He determined that the

rabbit macrophages produced a significant amount of thymidine kinase, but the mouse macrophages did not.

It was important to determine if another early enzyme, DNA polymerase was synthesized by the resistant cells. This enzyme is directly involved with DNA synthesis. DNA polymerase is produced in the susceptible macrophages infected with vaccinia virus after the second stage uncoating. This enzyme assay serves as an important marker which can provide additional information regarding early biochemical events which occur or do not occur in the non-permissive mouse macrophages as compared to the permissive rabbit macrophages.

DNA polymerase was assayed using a liquid scintillation counter which detected the amount of ^{32}P-ATP incorporated into the enzyme product, DNA. The results indicated that both mouse fibroblasts and rabbit macrophages, which are permissive for the vaccinia virus infection, produced the early enzymes DNA polymerase (Tables 1, 2, 3). However, there was no significant amount of DNA polymerase produced in the non-permissive mouse macrophages (Tables 2, 3). Therefore, it was concluded that the enzymes, thymidine kinase and DNA polymerase are not produced in the resistant mouse macrophages.

It was important to determine whether the second stage uncoating in the non-permissive mouse macrophages was viral induced in order to determine if the enzyme responsible for second stage uncoating was being synthesized in the resistant macrophages or if the uncoating was a result of cellular enzymes. The positive control system consisted of vaccinia virus infected mouse macrophages and the

experimental system consisted of vaccinia virus infected mouse macrophages treated with the protein synthesis inhibitor Streptovitacin A. Second stage uncoating was determined by measuring the amount of H^3-thymidine labeled viral DNA which became sensitive to DNase.

Second stage uncoating results revealed that when lysate samples were treated with DNase, the viral genome was degraded in the positive control system (Table 4). This was evident by the presence of a higher radioactive count in the supernatant of the positive control system. There was a 4 h lag before a significant radioactive count was observed. This sensitivity to viral DNA was observed up to 8 h (Table 4). However, the supernatant of control samples consistently showed low amounts of radioactive material in the absence of DNase (Table 4). These findings established the presence of second stage uncoating in the resistant mouse macrophages. This study, along with the findings of Kashanchi (1986) and Mpwo (1989) suggested the presence of second stage of uncoating in the resistant mouse macrophages. However, there was no occurrence of second stage uncoating in the mouse macrophages treated with the protein synthesis inhibitor Streptovitacin A. This was evident by the presence of lower radioactive material in the supernatant at 4 h and 8 h post infection (Table 5). The results of this study indicated that the host must synthesize protein at the time of infection for the release of viral DNA from the viral core. Also, this study revealed that the enzyme responsible for second stage uncoating is being synthesized in the non-permissive mouse macrophages.

Novikoff et al. (1964) proposed a different mechanism of second stage uncoating. They reported that the virus and the viral cores can be digested by the secretion of lysosomal enzymes. If the lysosomal enzymes are playing a role in the second stage uncoating, then the protein synthesis of the host at the time of inoculation is not necessary to release viral DNA (Dales, 1965).

Joklik (1963) did a study in order to examine whether release of DNA from vaccinia requires short-term protein synthesis of the host. He examined for the second stage uncoating in puromycin treated Hela cells infected with vaccinia virus. He discovered that the protein synthesis inhibitor decreased uncoating by 80 % to 90 %. On the other hand, he observed uncoating in the untreated Hela cells.

Later Dales (1965) supported the work of Joklik (1963) by doing a study on the effects of Streptovitacin A on the initial events in the replication of vaccinia and reovirus. He examined for the second stage uncoating of virus in Streptovitacin A treated Hela cells infected with vaccinia virus. The results of his study revealed that there was no second stage uncoating of virus in Streptovitacin A treated cells. However, there was second stage uncoating of virus in cells which were infected with vaccinia virus 5 h after removal of the protein synthesis inhibitor.

This study, along with the findings of Joklik (1963) and Dales (1965), suggest that the host must synthesize protein at the time of infection for the release of viral DNA from the viral core and that second stage uncoating in the resistant mouse macrophages was not due

to the secretion of lysosomal enzymes of the host. The viral protein acts as an inducing agent and activates the portion of the host-cell genome coding for the protein which cause the release of viral DNA (Joklik, 1963). Because the second stage uncoating in mouse macrophages was viral induced, these findings indicated that the enzyme responsible for second stage uncoating was synthesized in the resistant cells.

Based on these findings it appears that some transcription and translation takes place when DNA is still in the protection of its protein coat. However, after the release of viral DNA into the cytoplasm of the host cell, protein synthesis is inhibited. The mechanism of this inhibition remains unknown. In conclusion, the present study indicates that normal mouse peritoneal macrophages express innate resistance against vaccinia virus by suppressing the synthesis of early enzymes such as thymidine kinase and DNA polymerase which leads to suppression of viral DNA synthesis.

SUMMARY

DNA polymerase assay revealed no significant amount of DNA polymerase activity in mouse macrophages in comparison to rabbit macrophages and mouse fibroblasts. These data suggest inhibition of early virus-induced enzymes by mouse macrophages as a means of expressing innate resistance against vaccinia virus.

Biochemical tests revealed that viral DNA was converted to a DNase sensitive form in the resistant mouse macrophages, thus confirming the occurrence of second stage of uncoating. In contrast, no evidence was found of second stage uncoating in the resistant mouse macrophages treated with the protein synthesis inhibitor Streptovitacin A. These data indicate that the second stage uncoating in the mouse macrophages is virus induced, and that the enzyme responsible for second stage uncoating is synthesized in the non-permissive macrophages.

LITERATURE CITED

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Beard, J.W. and P.J. Rous. 1938. The fate of vaccinia virus on cultivation *in vitro* with Kupfer cells. J. Exp. Med. 67:883.

Buchmeier, N.A., S.K. Gee, F.A. Murphy, and W.E. Rawls. 1979. Abortive replication of vaccinia virus in activated rabbit macrophages. Infect. Immunology. 26:328.

Clark, V.E. 1984. Mechanisms of genetically endowed macrophage resistance. Unpbl. Masters Thesis, ESU, 55 pp.

Dales, S. 1963. The uptake and development of vaccinia virus in strain L cells followed with labelled viral deoxyribonucleic acid. J. Cell Biol. 18:51.

_____. 1965. Penetration of animal viruses into cells. Progr. Med. Virol. 7:1.

_____. 1965. Effects of Streptovitacin A on the initial events in the replication of vaccinia and neovirus. Proc. Natl. Acad. Sci. U.S. 54:462.

_____. and E.H. Mosbach. 1968. Vaccinia as a model for membrane biogenesis. Virology. 35:564.

Esteban, M., and D.H. Metz. 1973. Early virus protein synthesis in vaccinia-infected cells. J. Gen. Virol. 19:201.

Florman, A.C., and J.F. Enders. 1942. The effect of homologous antisenum and complement on the multiplication of vaccinia virus in rollertube cultures of blood-mononucleus cells. J. Immunology. 43:159.

Glasgow, L.A. 1965. Leukocytes and interferon in the host response to viral infections. I. Mouse leukocyte-produced interferon in vaccinia virus infection *in vitro*. J. Exp. Med. 121:1001.

Greer, B. 1973. Factors affecting uptake and replication of vaccinia virus in peritoneal macrophages *in vitro*. Unpubl. Masters Thesis, ESU, 79 pp.

Hirsch, M.S., B. Zisman and A.C. Allison. 1970. Macrophages and age dependent resistance to herpes simplex virus in mice. J. Immunology. 104:1160.

Holowezak, T.A., and W.A. Joklik. 1967. Studies on the proteins of vaccinia virus. I. Structural proteins of virions and cores. Virol. 33:717.

Hood, G. 1978. Immunology. The Benjamin/Cummings Publishing Company, Inc. 63.

Hymer, W.C. and E.L. Kuff. 1964. Isolation of nuclei from mammalian tissues through the use of Triton X-100. J. Histochem. Cytochem. 12:350.

Johnson, R.T. 1964. The pathogenesis of herpes virus eucephalitis. II. A cellular basis for the development of resistance with age. J. Exp. Med. 120:359.

Johnston, R.B. 1988. Monocytes and macrophages. New Eng. J. Med. 318:747.

Joklik, W.K. 1963. The intracellular uncoating of poxvirus DNA. II. The molecular basis of the uncoating process. J. Mol. Biol. 8:277.

_____. 1966. The Poxviruses. Bact. Rev. 30:33.

_____. 1968. The large DNA animal viruses: The pox virus and herpes virus group. In Molecular Basis of Virology. (H. Fraenkel-Convat, Ed.) Reinhold, New York.

Jungwirth, C., and W.K. Joklik. 1965. Studies on "Early" enzymes in Hela cells infected with vaccinia virus. Virology. 27:80.

Kashanci, F. 1986. A comparative study of early events in resistance and susceptible macrophages infected with vaccinia virus. Unpbl. Masters Thesis, ESU. 90 pp.

Langevoot, H.L., Z.A. Cohn, J.G. Hirsch, J.H. Humphrey, W.G. Spector, R. Van Furth. 1970. The nomenclature of mononuclear phagocytic cells. Proposal for a new classification. In Mononuclear Phagocytes. (R. Van Furth, Ed.) 1. Blackwell Scientific Publications, Oxford.

McAuslan, B.R. and J.R. Kates. 1966. Regulation of virus-induced deoxyribonucleases. Proc. Nat. Acad. Sci. U.S. 55:1581.

Metchnikoff, E. 1892. LeCons sur la pathologie comparie de l' inflammation. Cited in Mononuclear Phagocytes. (R. Van Furth, ed.) 1. Blackwell Scientific Publications, Oxford.

Milligan, G.N. 1983. A proposed mechanism for the inhibition of vaccinia virus by mouse macrophage. Unpbl. Masters Thesis, ESU. 69pp.

Moss, B. 1985. Replication of Poxviruses. In: Virology (B.N. Fields, ed.) Raven Press, New York.

Mpwo, M. 1989. Intracellular mechanisms of innate resistance in vaccinia virus. Unpbl. Masters Thesis, ESU. 83 pp.

Nishmi, M. and H. Bernkopf. 1958. Toxic effect of vaccinia virus on leukocytes in vitro. J. Immunology. 81:460.

_____ and H. Niecikowski. 1963. Interactions of vaccinia virus and cells in primary and continuous culture. Nature. 199:1117.

Novikoff, A.B., E. Essner and N. Quintana. 1964. Golgi apparatus and lysosomes. Federation Proc. 23:1010.

Penman, S., K. Scherrer, Y. Becker, and J.F. Darnell. 1963. Polyribosomes in normal and poliovirus-infected Hela cells and their relationship to messenger RNA. Proc. Natl. Acad. Sci. U.S. 49:654.

Rabinovitch, M. 1968. Seminars in Hematology. 5:134.

_____. 1970. Phagocytic recognition. Cited in Mononuclear Phagocytes. (R. Van Furth, ed.) 294. Blackwell Scientific Publications, Oxford.

Schultz, W.W. 1966. An in vitro study of the susceptibility of peritoneal macrophages from mice and rabbits to vaccinia virus. Unpbl. Masters Thesis, ESU. 81 pp.

Silverstein, S. 1970. Macrophages and viral immunity. Seminars in Hematology. 7:185.

Van Furth, R., H.L. Langevoort and A. Schaberg. 1975. Mononuclear phagocytes in human pathology-proposal for an approach to improved classification. In Mononuclear phagocytes in Immunity Infection and Pathology. (R. Van Furth, ed.) Blackwell Scientific Publication, Great Britain.

Ward, J.E. 1980. The interaction of stimulated mouse macrophages with vaccinia virus in vitro. Unpbl. Masters Thesis, ESU, 50 pp.

Weiss, L. 1972. The cells and tissues of the immune system. Structure, functions, interactions. (A.G. Olsen and L. Weiss, eds.). Prentice-Hall, Inc. New Jersey. 252 p.

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The Study of DNA Polymerase in Resistant and Susceptible Macrophages Infected with Vaccinia Virus

Title of Thesis

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Date Received