AN ABSTRACT OF THE THESIS OF

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Title: A Study of Factors Which Affect the Resistance of Alveolar Macrophages to Vaccinia Virus

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In vitro systems of pulmonary-alveolar macrophages from normal and vaccinia-immunized rabbits and their counterparts from rabbits stimulated by complete Freund's adjuvant were tested for their resistance to the IHD strain of vaccinia virus. It was found that alveolar macrophages could not be induced, either by vaccination or stimulation of the donor animals, to suppress the multiplication of the virus. This finding sets the alveolar macrophages apart from the peritoneal macrophages which do develop the ability to inhibit vaccinia virus when their donors are similarly treated. However, the alveolar macrophages harvested from adjuvant-stimulated animals resisted viral injury even though viral replication was taking place. This suggests that an enhanced physiological state of the alveolar macrophages is important in protecting the cells from vaccinia cytopathology. Because peritoneal macrophages develop resistance, co-incubation of the peritoneal macrophages and alveolar macrophages was done to test the possibility that interaction between two cell types could protect the entire population. This did not occur. In fact, the virus was not inhibited in the mixed macrophage culture. It was concluded that the protective mechanism of the host against a respiratory-originated viral infection is a combination of

humoral and cellular immunity and the exact role of the alveolar macrophages in pox viral infection remains to be defined.

A Study of Factors Which Affect the Resistance of Alveolar Macrophages to Vaccinia Virus

A Thesis

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INTRODUCTION

The pulmonary-alveolar macrophages (PAMs) are the resident mononuclear phagocytes of the lungs and function as the primary defense against inhaled particulate matter (1, 2). Since 1961, when Myrvik (3) described a technique to obtain PAMs by lung lavage, many studies have been performed to elucidate the details of the PAMs' function and their role in cell-mediated immunity in humans and animals.

Tissue macrophages of different organs are derived from progenitors in the bone-marrow (4). In animals given whole-body irradiation followed by bone-marrow graft, the PAMs were replaced by cells of donor origin. These studies have used radioisotopes (5), surface enzymes (4) or cytomegalovirus (6) as markers to distinguish between macrophages of host and donor origin. According to some investigators, the direct precursors of PAMs appear to be interstitial cells originating from circulating monocytes, which, in turn, arise from bone-marrow (7, 8). It may be in the interstitium that PAMs develop the functional and metabolic features different from blood monocytes (9, 10). Until recently, the ability of PAMs to self-replicate was questioned, but this has now been established in rodents (10, 11).

The morphological features of the PAMs obtained by pulmonary lavage have been studied by light and electron microscopy (3). The PAMs are of variable size (15-50μm) with slightly basophilic cytoplasm. The nuclei vary from round to deeply indented with fine chromatin. The cytoplasm may contain dust, erythrocytes, cell debris and bacteria, usually associated with phase-dense granules and vacuoles. The most clearly defined feature for differentiating PAMs from lymphocytes is

the ease with which PAMs form a monolayer on glass (8).

Ultrastructural study indicates that heterogeneity of the PAM is largely dependent on the phase of cellular activity (12). Generally, a PAM contains numerous mitochondria, endoplasmic reticulum, and many ribosomes. The dense bodies and vesicles within the PAM have a lamellar and lattice configuration. These vesicles probably present cellular debris phagocytized from the alveolar lumen (12).

The surface features of PAMs have been studied by scanning electron microscopy, and these studies demonstrated that the cell-surface membrane is highly ruffled (12). Another study, using human bronchopulmonary lavage, has shown two types of PAMs: the flat cell, which spread and has many pseudopods, and the round cell (13). Although both types were firmly adherent to a plastic surface, the flat cells were more active in phagocytosis.

The aerobic environment of the alveolus has stimulated PAMs to develop special metabolic adaptations. The freshly harvested PAMs contain a high level of cytochrome oxidase and a low level of pyruvate kinase and phosphofructokinase, which indicates a predominantly aerobic energy metabolism (12). It has been demonstrated that PAMs function at a relatively high oxygen tension and are oxygen-dependent for killing Staphylococcus aureus (14). Although PAMs are oxygen-dependent and have a high respiratory rate in contrast to other tissue macrophages, only a slight increase in oxygen consumption and hexosemonophosphate shunt activity are detectable during phagocytosis (15).

Metabolic inhibitors have been used to study the cellular and biochemical events during particle ingestion (14). Incubation of PAMs

under anaerobic conditions or during the use of inhibitors of glycolysis or oxidative metabolism partially inhibited phagocytosis (16). Therefore, PAMs depend on both glycolytic and oxidative metabolism during particle ingestion.

The physiology of phagocytosis and antimicrobial activity of PAMs and their surface-membrane receptors for various serum components have also been investigated. Daughaday and Dauglas (17) reported that PAMs have receptor sites for the crystallizable fragment (Fc) of IgG and for the third component of complement (C_3). They also demonstrated that IgG receptors are inhibited by aggregated IgG, but not by free immunoglobulin and that C_3 receptors occupy a portion of the membrane functionally independent of IgG receptors (17). In addition, it has been reported that IgG opsonized bacteria were phagocytized more readily than IgA-coated bacteria (18).

The bactericidal mechanism of PAMs is poorly understood, but oxidative metabolism appears to be involved in this activity. PAMs appear to generate H_2O_2 (14) but, in contrast to polymorphonuclears (PMN), peroxidase does not contribute in a major way to microbial killing by PAMs. Since the presence of superoxide (O_2^-) has been established by nitro blue tetrazolium reduction (15), the study suggested that H_2O_2 will react with O_2^- in the presence of superoxide dismutase to yeild $OH^{\cdot}$, which is a highly reactive radical and possesses an antibacterial property (14).

In addition to oxidative metabolism, lysosomal enzymes contribute to the antibacterial activity of PAMs. Evidence from radiolabeling and EM study has shown that the site of the synthesis of lysosomal enzymes

is the Golgi complex, from which enzymes are released into the cytoplasm as primary lysosomes. These primary lysosomes, in turn, will fuse to phagocytic vesicles to form secondary lysosomes (19).

Antimicrobial activity of PAMs can be enhanced by in vivo activation with a variety of agents. Sarber and colleagues (20) reported that Bacillus-Calmette-Guerin (BCG) vaccination elevated the concentration of the lysosomal enzymes acid phosphatase, p-glucuronidase, and lysozyme by 1.94, 1.42 and 2.51 fold, respectively. Moore and Myrvik (21) demonstrated that the BCG-vaccination induced lung cells to elaborate factors which inhibited the migration of normal PAMs. They also established that PAMs have receptors for migration inhibition factor (MIF), which is important in cell-mediated immunity and cell-mediated hypersensitivity. Horwitz and Crystal (22) were able to detect the presence of collagenase in PAMs and showed it increased after BCG-vaccination. PAMs are also able to synthesize and release a low molecular weight factor (less than 5000 d), with chemotactic properties for PMNs (23, 24). Mantarrosa and Myrvik (25) reported that activation of PAMs with complete Freund's adjuvant (CFA) increased the number of IgG receptors about 20%. Meanwhile CFA-stimulated PAMs exhibited a highly developed endoplasmic reticulum, a large accumulation of electron dense granules, and a highly ruffled cytoplasmic membrane (25).

Although PAMs' antimicrobial activity has been well studied, their antiviral mechanism has not been fully investigated. Studies with mousepox have shown that virus was taken up by PAMs and that it grew in at least some of them. The investigators believed that infected macrophages were the primary factors in dissemination of mousepox virus (26).

Using West Nile virus, Nir and colleagues (27) showed that viruses were detectable within the first 24 hours in PAMs cytoplasm and later in other cells. Warshauer and colleagues (28) suggested that influenza virus infection renders the PAMs susceptible to secondary infection because of the impairment of the antimicrobial activity of PAMs. Other groups of investigators (29, 30, 31, 32) have used both dermatropic and neurotropic strains of vaccinia virus and have demonstrated the inability of PAMs, from both normal and immune rabbits, to suppress viral replication *in vivo* and *in vitro*.

In the present study, the role of rabbit PAMs in vaccinia infection and their ability to support or suppress the International Health Division (IHD) strain of vaccinia virus have been investigated. This study has been performed in three steps. First, the interaction of vaccinia virus with PAMs from normal and vaccinia-immunized rabbits was studied to establish a base line for comparison of viral activity in the next two sections. The second step was undertaken to study the ability of CFA-stimulated PAMs, with their marked changes, to support or suppress the vaccinia replication. And, finally, the study examines the course of viral activity in the mixture of lung and peritoneal macrophages in equal population per ml. The purpose of the last step was to detect whether or not co-incubation of PAMs with a known suppressor of vaccinia virus could cause supression of viral replications in the PAMs.

MATERIALS AND METHODS

Experimental Animals

Male and female New Zealand white rabbits 3-6 months old were purchased from a local breeder. White mice were supplied by the Emporia State University animal room.

Tissue Culture Media

Cells were grown in Eagle's Minimum Essential Medium (MEM) containing L-glutamine (Gibco). This medium was used as a maintenance medium or a complete medium as well as an infecting medium. Hank's balanced salt solution (HBSS) with heparin (5 IU/ml) and a combiotic was used in harvesting both PAMs and PMs. HBSS without heparin was used to wash and seed the macrophages. Dulbecco's phosphate buffer solution was used in two forms, one without Mg^{+2} and Ca^{+2} ($D\text{-}PBS^{-}$) and the other with both divalent cations (D-PBS). $D\text{-}PBS^{-}$ was employed to harvest mouse-embryo fibroblasts, and D-PBS was used to wash cell monolayers, fibroblasts, and macrophages before infection.

A stock solution of combiotic consisting of penicillin G (Gibco, 1650 U/mg) and streptomycin sulfate (Schwartz/Mann) was made in deionized glass-distilled water, sterilized by membrane (0.45 μm) filtration, aliquoted in one ml quantities, and stored at -20 C. Combiotic at a final concentration of penicillin 200 IU/ml and streptomycin 100 IU/ml was used in all solutions except the trypsinizing solution.

A stock solution of trypsin (Difco, 1:250) 5% w/v was prepared in $D\text{-}PBS^{-}$ or deionized water, membrane filtered, aliquoted in 5 ml quantities, and stored at -20 C. Ethylendiaminetetraacetate (EDTA) 10% w/v was made, sterilized, and stored in one ml quantities at -20 C. The EDTA was used

to chelate Ca^{+2}, which interferes with trypsin action. The trypsinizing solution contained the final concentration of trypsin 0.25% and EDTA 0.02%.

Fetal calf serum (FCS) was obtained from Colorado Serum Co. FCS was inactivated at 56 C for 30 minutes and stored at 4 C.

Harvesting Mouse-Embryo Fibroblasts

Fourteen-to-seventeen day old embryos were harvested and placed in a sterile glass petri dish. The embryos were decapitated, eviscerated and washed in two consecutive changes of $D\text{-}PBS^{-}$. After washing, the embryos were minced, and the minced tissue was placed into a trypsinizing flask containing 100-150 ml trypsinizing solution. Trypsinization was interrupted every 15-20 minutes and the cell suspension was poured off and passed through cheese-cloth into D-PBS with 10% FCS. The decanted solution was replaced by 100-150 ml fresh trypsinizing solution and this process was then repeated three more times. Cells were pelleted by centrifugation (500g for 10 minutes). The supernatant was poured off and cells were resuspended in D-PBS and centrifuged again. This procedure was followed by final resuspension of cells in a known volume of MEM with 10% FCS. The cell count was adjusted to $2X10^{6}$ cell/ml and planted in Roux flasks or Corning tissue culture flasks in 60 and 5 ml quantities, respectively.

Virus: Propagation

Roux flasks, containing 60 ml cell suspension, were incubated at 37 C for 48-72 hours until the cells were almost 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^{4} plaque forming unit (PFU)

of IHD vaccinia per ml. After 2 hour adsorption at 37 C, 50 ml of MEM with 2% FCS was added to the Roux flasks which were incubated at 37 C for 48-72 hours. After 72 hours, extensive cell damage indicated viral replication, after which Roux flasks were stored at -70 C.

Virus: Viral Inoculum

Roux flasks containing the virus were allowed to thaw at room temperature with frequent agitation to remove cells from the flask walls. The resulting suspension was placed in a treatment unit of a Raytheon Oscillator (Model DF-101) and sonified at 0.9 ampere for 1-1.5 minutes to break the cells and release the virus. After centrifugation (1300g for 10 minutes), the supernatant was collected, aliquoted in small quantities, and stored at -70 C.

Virus: Assay of Virus

Ten-fold serial dilutions of virus to be titered were prepared in MEM with 2% FCS. One ml of each dilution was inoculated in a plastic tissue culture flask containing mouse fibroblast monolayer, which had been washed two times with D-PBS. After incubation at 37 C for 2 hours, 4 ml of MEM with 2% FCS were added to each flask, and each flask was incubated at 37 C for 44-48 hours. Then the supernatant was poured off and the monolayer stained for 5 minutes with Hucker's modification of crystal violet diluted 1:10. Stained flasks were washed with tap water and air dried. Plaques were counted and the viral titer was expressed as plaque forming units per milliliter (PFU/ml).

Immunization Procedure

The rabbit's back was shaved and five intradermal injections of 0.1 ml of IHD vaccinia ($4X10^8$ PFU/ml) were administered. Approximately two

weeks following vaccination, animals received 2 ml vaccinia ($4X10^8$ PFU/ml) intravenously via the marginal ear vein. Injections were repeated every 2-3 weeks, and the last injection was administered 6 days before sacrifice.

Complete Freund's adjuvant injection, 0.2 ml intravenously, was followed within 48 hours by the last injection of virus. Control animals received the CFA only. Rabbits were sacrificed two weeks after the CFA injection.

Harvesting PAMs

Rabbits were sacrificed with an air embolism produced by injecting 50 ml air intravenously. The chest was disinfected, washed and shaved. The skin was cleaned and disinfected, and laid back to expose the muscles. An incision was made to expose the trachea, which was then clamped below the larynx. The rib cage was dissected away and the lungs removed. After removing extraneous tissues, the lungs were wrapped in cheese-cloth moistened with disinfectant. Fifty ml of HBSS with heparin and combiotic was used to fill the lungs, which were then gently massaged for 1-2 minutes and HBSS containing cells was retrieved. Washing was repeated two more times and suspensions were placed in centrifuge tubes and centrifuged (500g for 10 minutes). The supernatant was decanted and cell pellets were resuspended in a known volume of HBSS. Cells were adjusted to $3X10^6$ cell/ml and one ml quantities were planted in coverslip tubes, which were then incubated at 37 C for 1-1.5 hours.

Harvesting PMs

Both immune and normal rabbits, which had received 50 ml sterile mineral oil intraperitoneally six days before harvesting, were sacrificed

by an embolism. The abdomen was disinfected and shaved, and the skin laid back exposing the muscles around the peritoneal cavity. An incision of 3-5 cm was made and the peritoneal cavity was washed with 200 ml HBSS with heparin and combiotic. The oil - HBSS - cell suspension was collected, placed into a separatory funnel, and kept at 4 C for 15 minutes to allow the separation of mineral oil. After 15 minutes, the aqueous phase, consisting of HBSS and cells, was poured into a centrifuge tube and centrifuged (500g for 10 minutes). The supernatant was Decanted, the cell pellets resuspended and centrifuged again. The washing was carried out one more time. The cells were then resuspended in a known volume of HBSS, adjusted to $3X10^6$ cell/ml and seeded in 1 ml quantities into the coverslip tubes. Tubes were incubated at 37 C for 1-1.5 hours.

Mixed Population of PAMs and PMs

PAMs and PMs were obtained as described. The two cell types were mixed to a final concentration of $3X10^6$ cell/ml with equal populations of each type. The cell mixture was planted into the coverslip tubes and incubated at 37 C for 1-1.5 hours. Then HBSS was removed, the cell monolayer was washed, the complete medium (MEM with 10% FCS) added, and the cells incubated at 37 C for 20-24 hours.

Staining Procedure

Coverslips were removed from coverslip tubes, air dried, and fixed in absolute methanol for 5 minutes. After fixation, they were stained in May-Grunwald for 9 minutes and Giemsal for 14 minutes. Then they were dehydrated in acetone, rinsed in a 50/50 solution of acetone and xylene, and cleared in xylene for 10 minutes. Finally, coverslips were

mounted on glass slides.

Infection of Macrophages

After 1-1.5 hours incubation, the HBSS was removed and cell monolayers were washed two times with D-PBS. At this time tubes 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 with 2% FCS and $3X10^6$ PFU/ml IHD vaccinia virus. The control tubes received only MEM with 2% FCS. A sample of infecting medium was held and titered to establish the zero hour viral activity. After two hours of incubation, the supernatant was removed and monolayers were washed two times with D-PBS to remove any unadsorbed virus particles. The last washing was followed by the addition of 1 ml of the complete medium (MEM with 10% FCS) to each tube. Infected as well as control tubes were treated in the same way throughout this procedure. At this time, 2 hour post-infection, duplicate samples of infected tubes were taken to be titered for the amount of virus which had been adsorbed. At 24 hour intervals, samples were taken which were then kept at -70 C until titered. At the same time coverslips from control and infected tubes were stained for light microscopic examination. The above described procedure was also employed to infect the mixed culture of the PAMs and PMs.

Autoradiography

After two hour adsorption, the macrophage monolayers were washed with D-PBS and the complete medium containing 1 uCi/ml of tritiated thymidine (New England Nuclear, specific activity 66.6 mCi/mmol) was

added to both infected and control systems. Duplicate samples of control and infected macrophages were taken 8 hours following infection and then every 24 hours.

The coverslips were air dried and fixed in absolute methanol for 5 minutes. After fixation, coverslips were placed in a cold solution of 2% perchloric acid for 15-20 minutes, then were washed in distilled water for 30 minutes. Then, the back of the coverslips were gently cleaned and mounted cell side up on a glass slide and air dried. The dry slides were dipped in a solution of Chrom-Alum Gelatin (0.1% chrom-alum and 1% gelatin) and air dried.

In the dark room, the film emulsion (Kodak NTB2) was liquified in a water bath at 45-50 C. Prewarmed slides were dipped into the emulsion, withdrawn immediately, and shaken to remove extra emulsion. This left a thin layer of emulsion on the slides. The slides were then dried in an air current, placed in light-tight boxes containing dehumidifer, and stored at 4 C for 10 days.

At the end of the exposure period, the slides were developed in D-19 solution (Kodak) for 10 minutes, briefly washed in distilled water, placed in Kodak fixative for 15 minutes, and washed in several changes of water for 30 minutes. Washed slides were stained in Giemsa for 15 minutes, rinsed in water, and counter-stained with Delafield's hematoxylin for 1.5 minutes and with saturated lithium carbonate for 1 minute. The slides were then dehydrated in increasing concentrations of methanol, cleared in xylene for 5-10 minutes, and cover-slipped with permount.

RESULTS

PAMs from both normal (N-PAM) and vaccinia-immunized (I-PAM) rabbits were infected *in vitro* with the IHD strain of vaccinia virus ($3X10^6$ PFU/ml). The cells adsorbed approximately the same amount of virus after *in vitro* infection, followed by viral replication in both cell types (Fig. 1).

The course of the viral replication was monitored every 24 hours up to 96 hours post-inoculation. The virus had multiplied in both cell types by 24 hours and reached its peak 48 hours post-infection. After this point, N-PAMs continued to support the viral replication and viral titer increased, but only very slightly. The I-PAMs showed a very similar increase in viral titer up to 72 hours, but the titer declined at 96 hours.

Light microscopic studies of the May-Grunwald Giemsa-stained coverslips from infected N-PAM and I-PAM along with their control samples revealed no morphological differences between control and infected cells at two hour post-inoculation (Fig. 2-5). The first evidence of cytopathic effect (CPE), which was expressed as cell aggregation, was observed at 24 hours in both infected cell types (Fig. 6-7). Although infected N-PAMs and I-PAMs showed some signs of viral infection, only N-PAMs demonstrated extensive CPE at this time interval. At 48 hours, many of both types of cells had lost their typical round morphology and some cell destruction was noted. At 72 and 96 hours, gross cell destruction and cell loss from coverslips were noticed in both N-PAMs and I-PAMs (Fig. 8-9).

Some binucleated giant cells were observed at 2 hour post-infection

and by 24 hours, the population of these cells and multinucleated cells had increased significantly. Up to 48 hours, as well as after 48 hours, they had been injured like other cells and it became impossible to detect these giant cells in the aggregated cells on the coverslips.

Autoradiographic examination of the unstimulated normal and immune PAMs showed that tritiated thymidine (^{3}H-TdR) incorporation into the viral DNA in PAMs' cytoplasm increased in conjunction with the viral titer (Fig. 10). There was some incorporation of ^{3}H-TdR into the PAMs' nuclei at the level of 6-7% of the population throughout the experiment, but this activity was not considered to be the site of the viral replication, which takes place exclusively in the cytoplasm. The first ^{3}H-TdR incorporation into the viral DNA was detected in the samples taken at 8 hour post-inocultaion. This activity was manifested by both the infected PAMs at the level of 3-4% (Fig. 11-12). The cytoplasmic activity had increased to 28-30% of the population in both infected cell types by 24 hours (Fig. 13-14). Another marked increase in the cytoplasmic accumulation of ^{3}H-TdR was observed at 48 hours. At this time, at least 80% of the I-PAMs and 50% of the N-PAMs showed cytoplasmic inclusions of ^{3}H-TdR (Fig. 15-16). After this time many cells had detached from coverslips due to the extensive cell injury, and most of the remaining cells contained clumps of ^{3}H-TdR in their cytoplasm.

Autoradiographic studies of the control system revealed that at 8 and 24 hours after infection there was a slight cytoplasmic ^{3}H-TdR activity of about 1% in both normal and immune controls (Fig. 10, 17-18). Cytoplasmic activity had reached its peak by 48 hours, and by 72 and 96 hours cytoplasmic activity had declined to the level of 1%.

Figure 1. Viral titers as a function of time in normal (N-PAM) and immune (I-PAM) macrophages infected with vaccinia. PFU = plaque forming units.

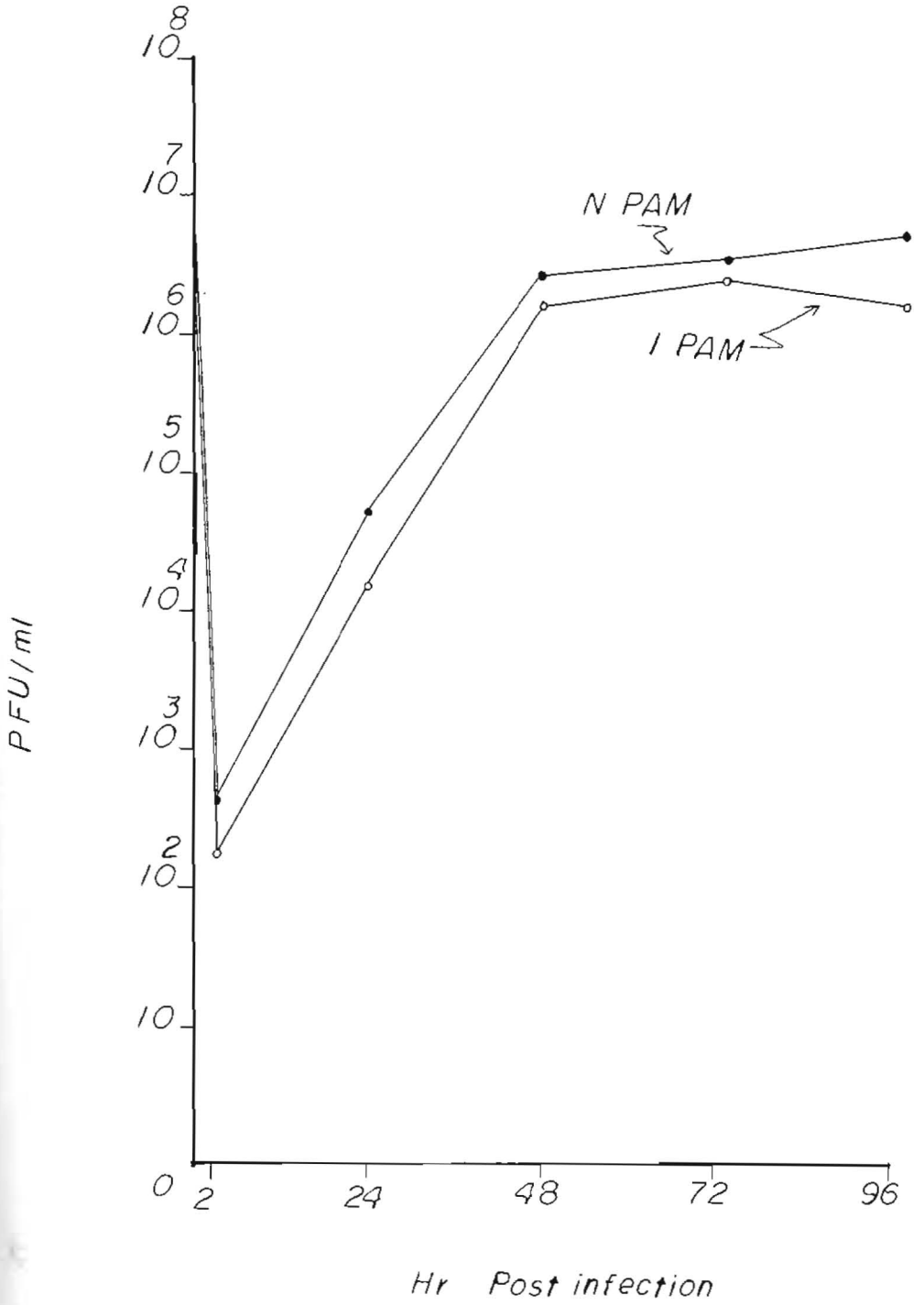
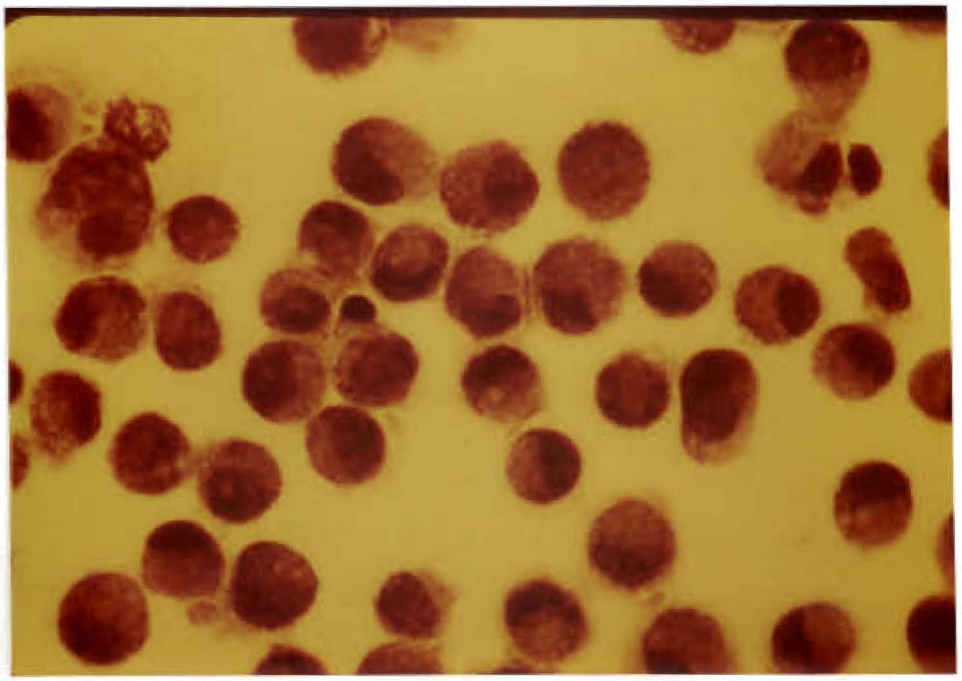

PFU/ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
N PAM
I PAM
2
24
48
72
96
Hr Post infection

Figure 2. Uninfected normal rabbit PAMs at 2 hours (X 400).

Figure 3. Normal rabbit PAMs infected for 2 hours (X 400). No change due to viral infection.



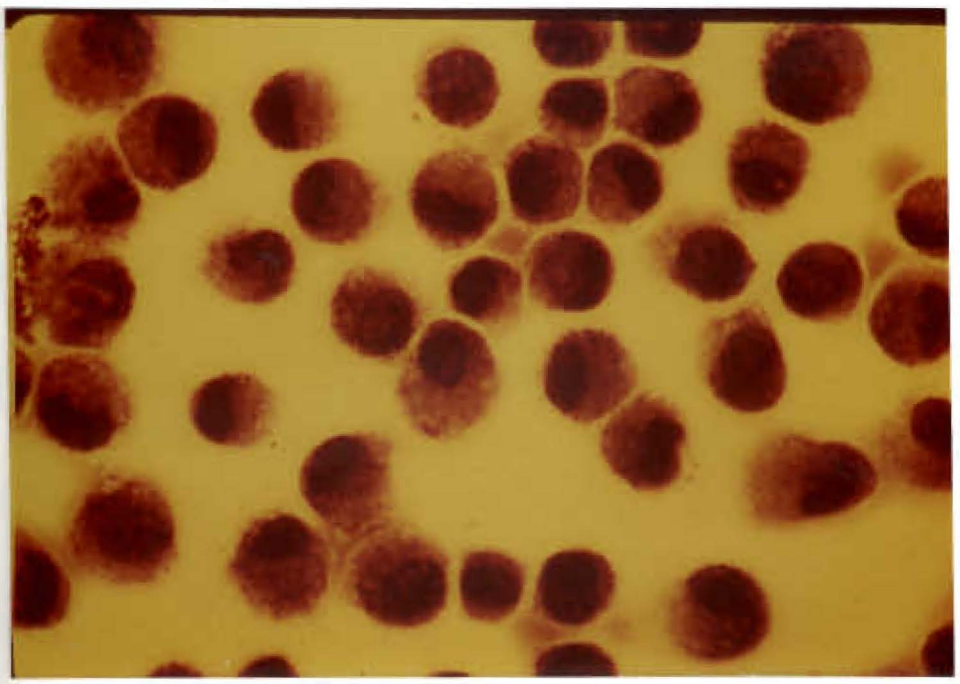
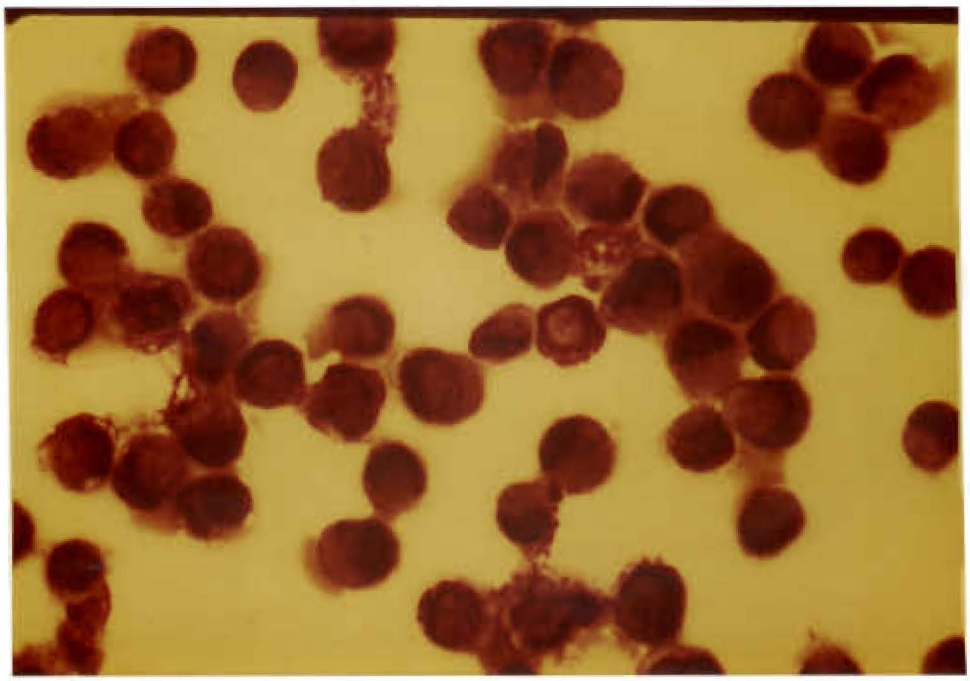


Figure 4. Uninfected immune rabbit PAMs at 2 hours (X 400).

Figure 5. Immune rabbit PAMs infected for 2 hours (X 400). No change due to viral infection



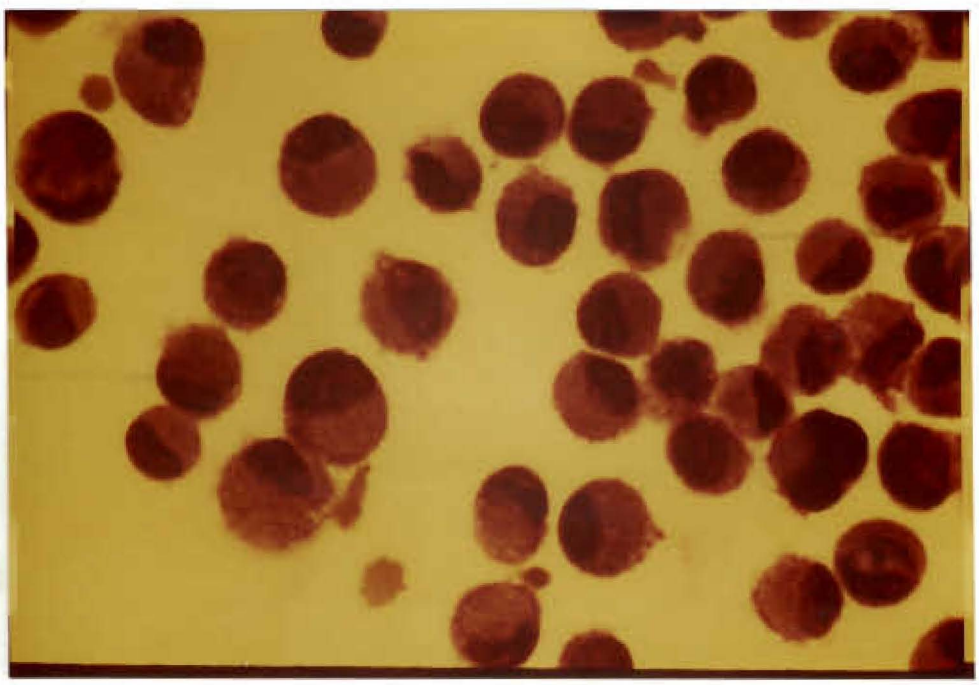
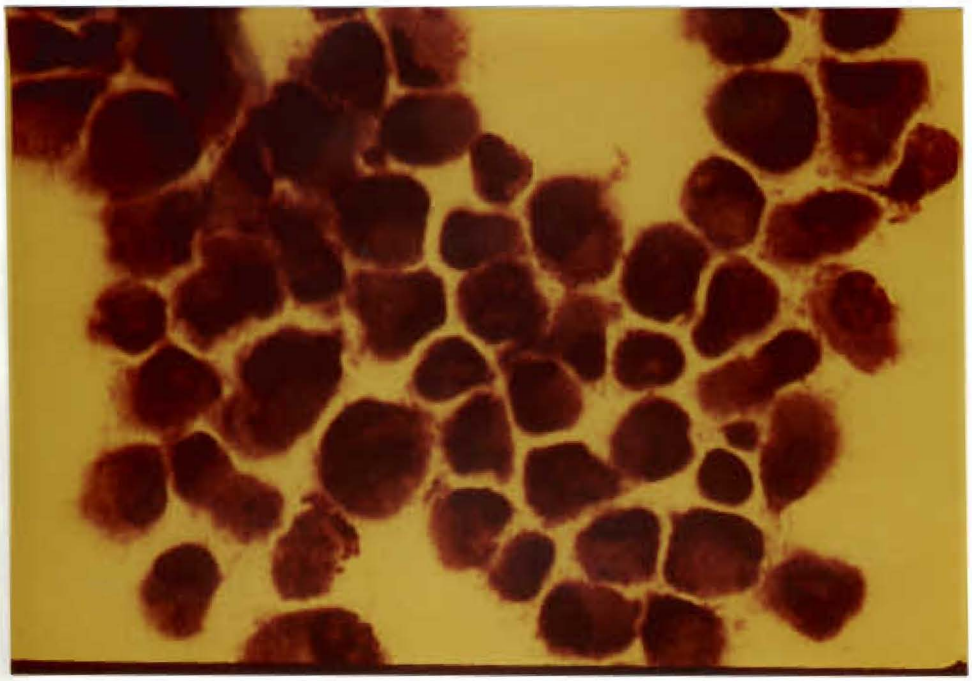


Figure 6. Normal rabbit PAMs infected for 24 hours (X 400). Showing cell aggregation due to virus.

Figure 7. Immune rabbit PAMs infected for 24 hours (X 400). Showing cell aggregation comparable to that in normal PAMs.



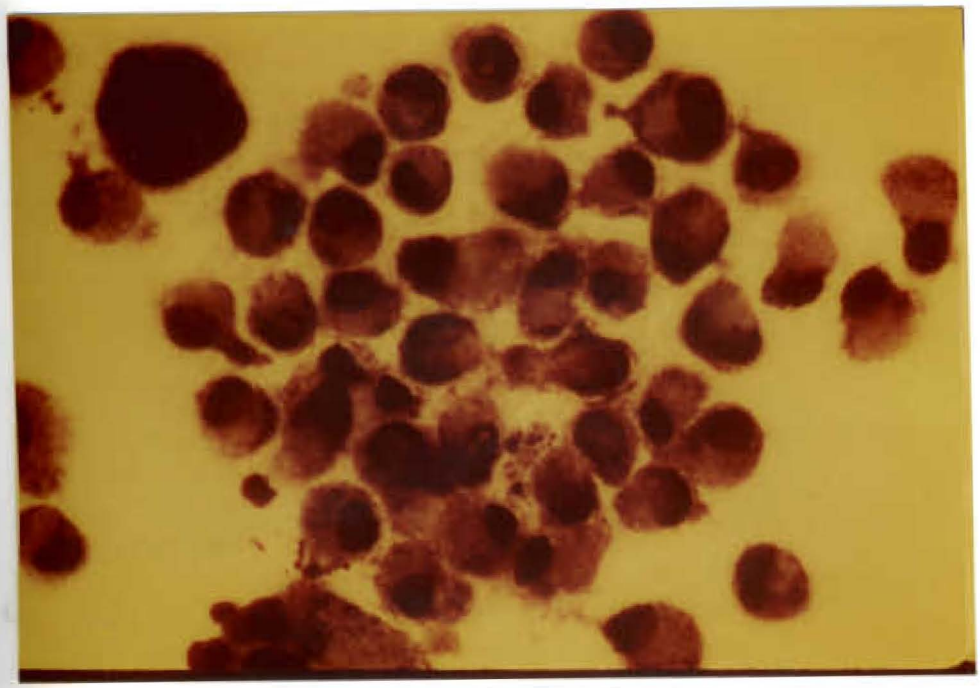
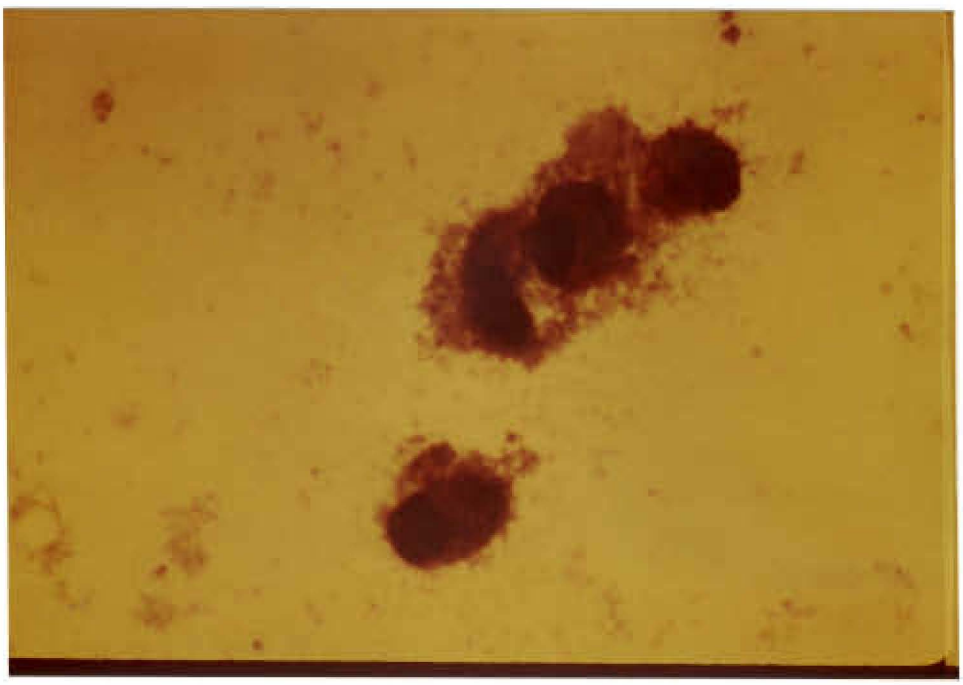


Figure 8. Normal rabbit PAMs infected for 96 hours (X 400). Illustrating extensive viral injury.

Figure 9. Immune rabbit PAMs infected for 96 hours (X 400). Exhibiting cytopathology equivalent to that in the infected normal PAMs.



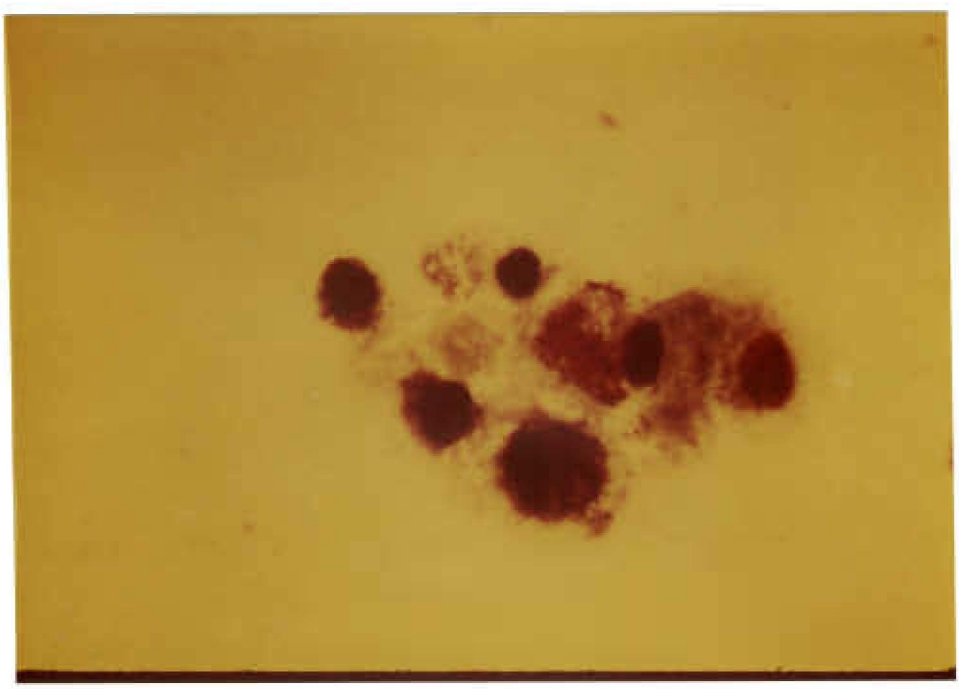


Figure 10. Time course of viral induced DNA synthesis in immune (I-PAM) and normal (N-PAM) macrophages infected with vaccinia *in vitro*.

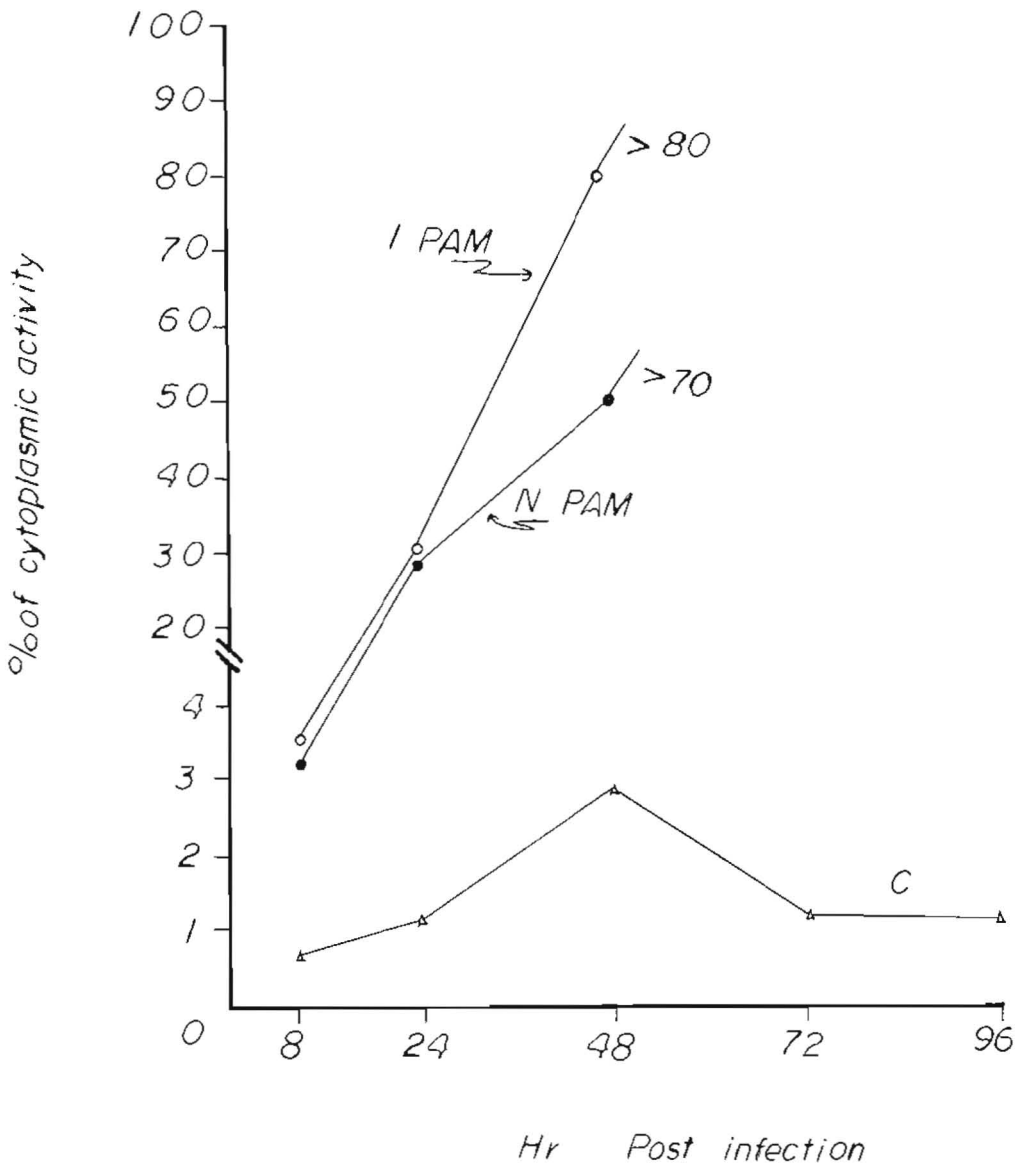
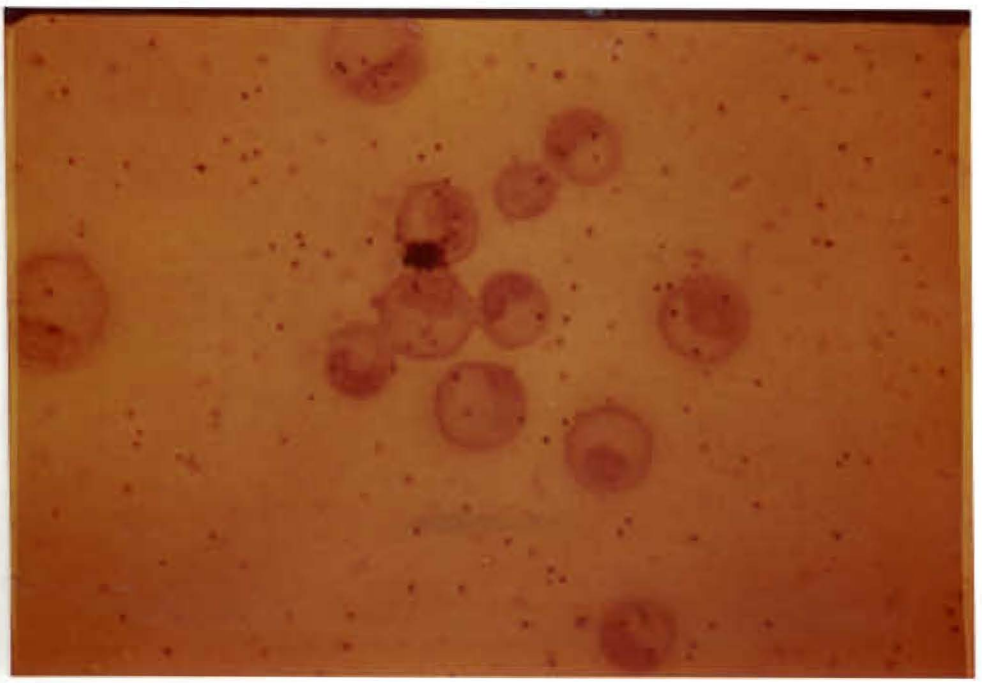

100
90
80
70
60
50
40
30
20
4
3
2
1
0
8
24
48
72
96
> 80
1 PAM
> 70
N PAM
C
% of cytoplasmic activity
Hr Post infection

Figure 11. Autoradiograph of normal rabbit PAMs infected for 8 hours (X 400). Showing the typical viral induced DNA synthesis.

Figure 12. Autoradiograph of immune rabbit PAMs infected for 8 hours (X 400) with typical viral induced cytoplasmic activity.



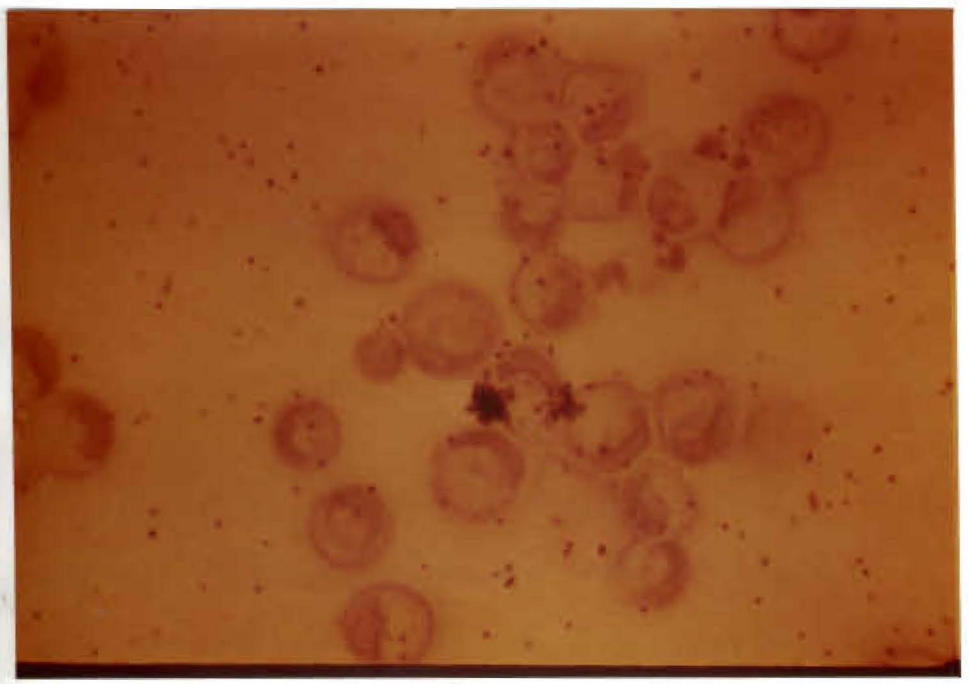
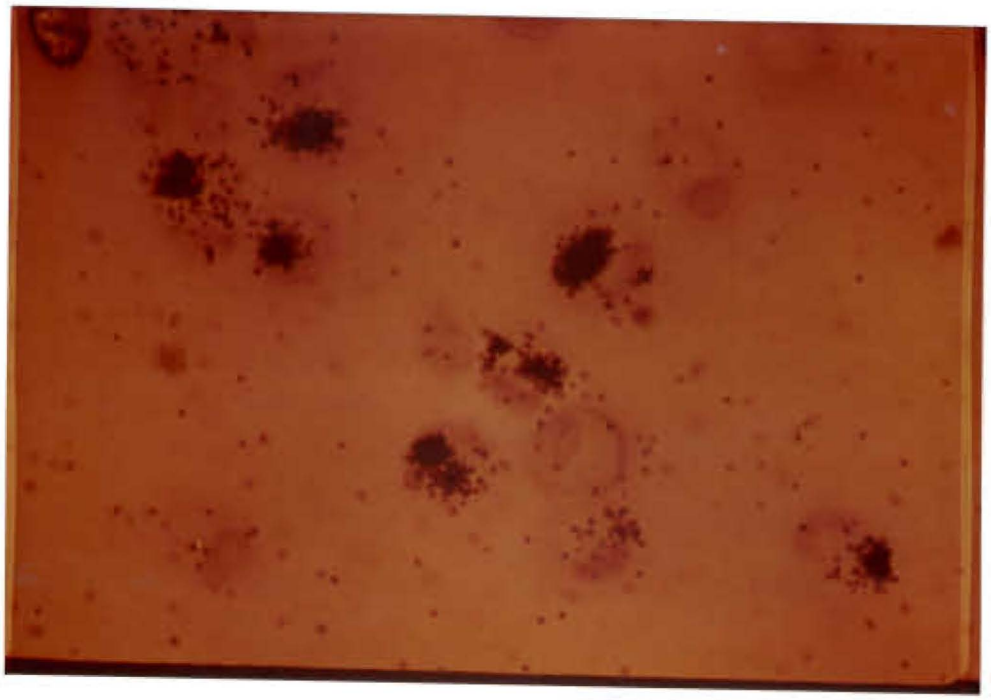


Figure 13. Autoradiograph of normal rabbit PAMs infected for 24 hours (X 400). Showing the viral induced DNA synthesis increased with time.

Figure 14. Autoradiograph of immune rabbit PAMs infected for 24 hours (X 400). Showing the viral induced DNA synthesis increased with time.



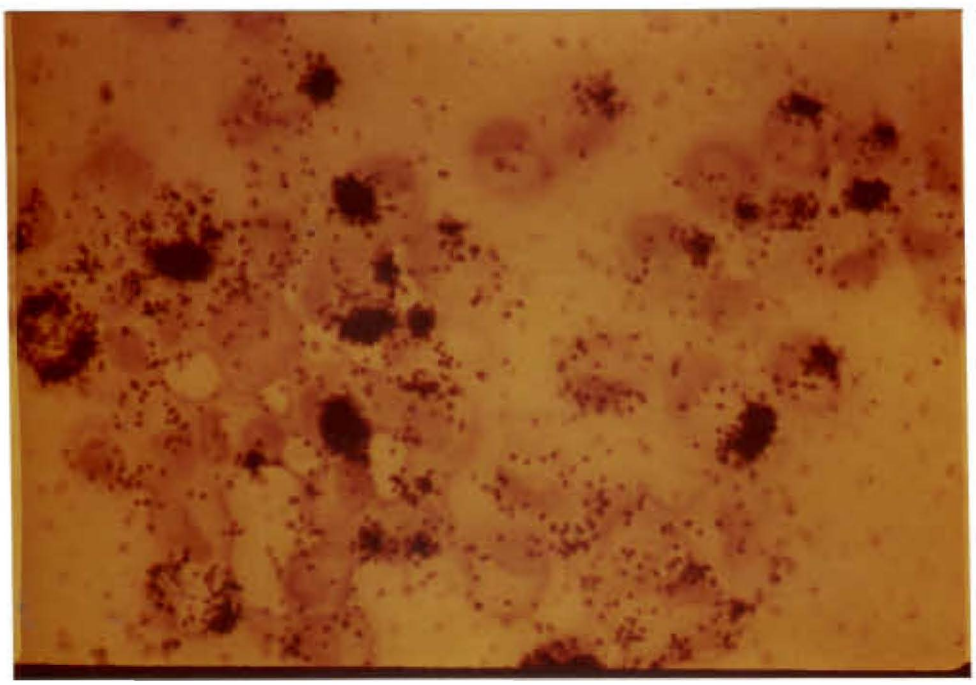
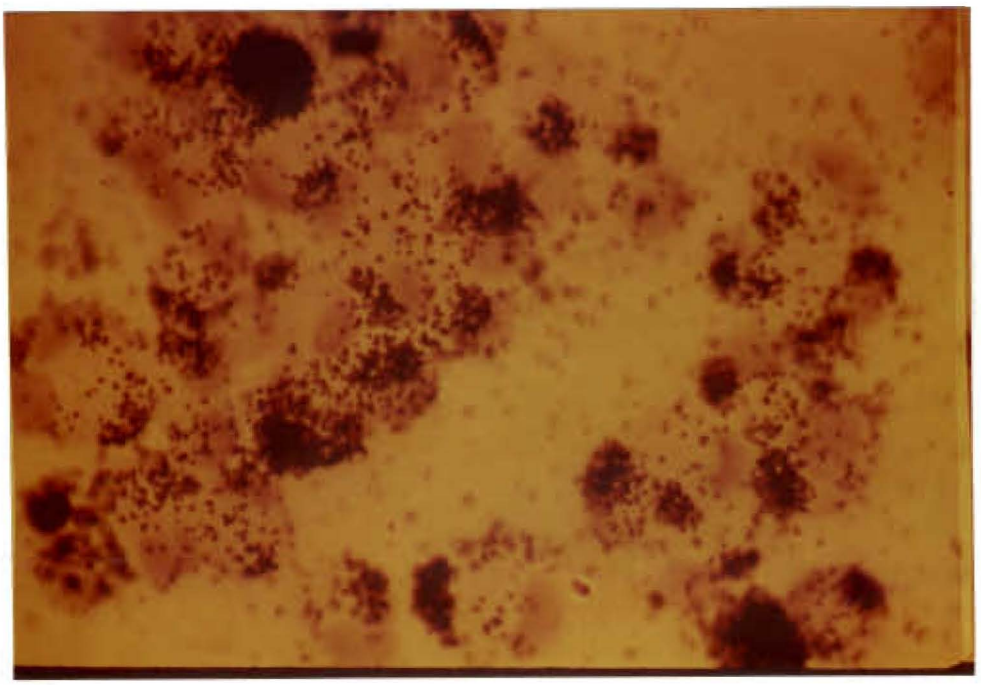


Figure 15. Autoradiograph of normal rabbit PAMs infected for 48 hours (X 400) with extensive cytoplasmic activity indicating spread of viral infection.

Figure 16. Autoradiograph of immune rabbit PAMs infected for 40 hours (X 400). Showing activity comparable to that of normal controls.



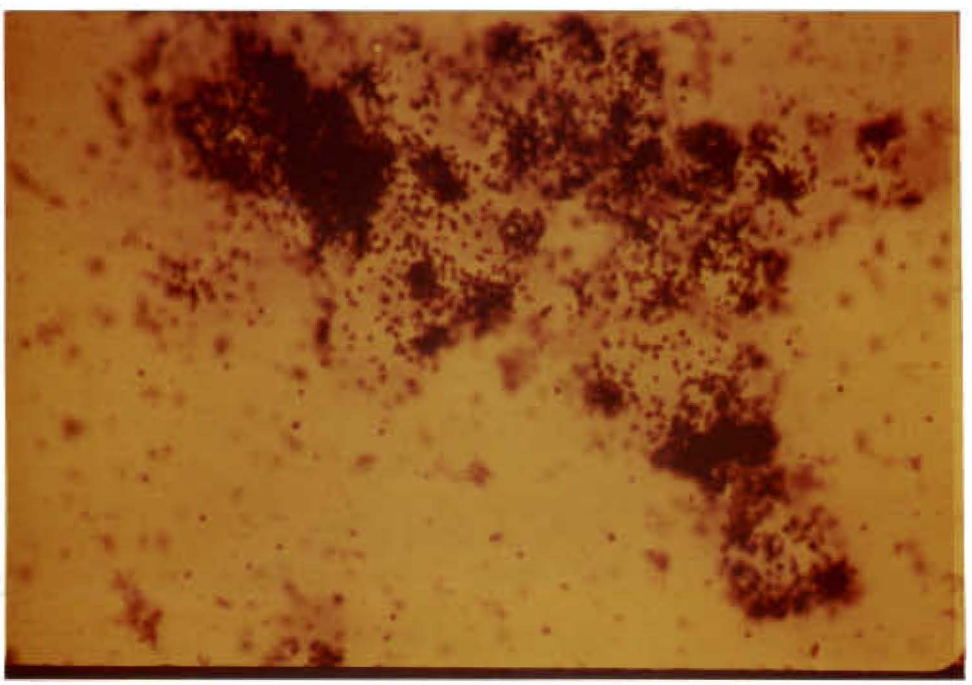
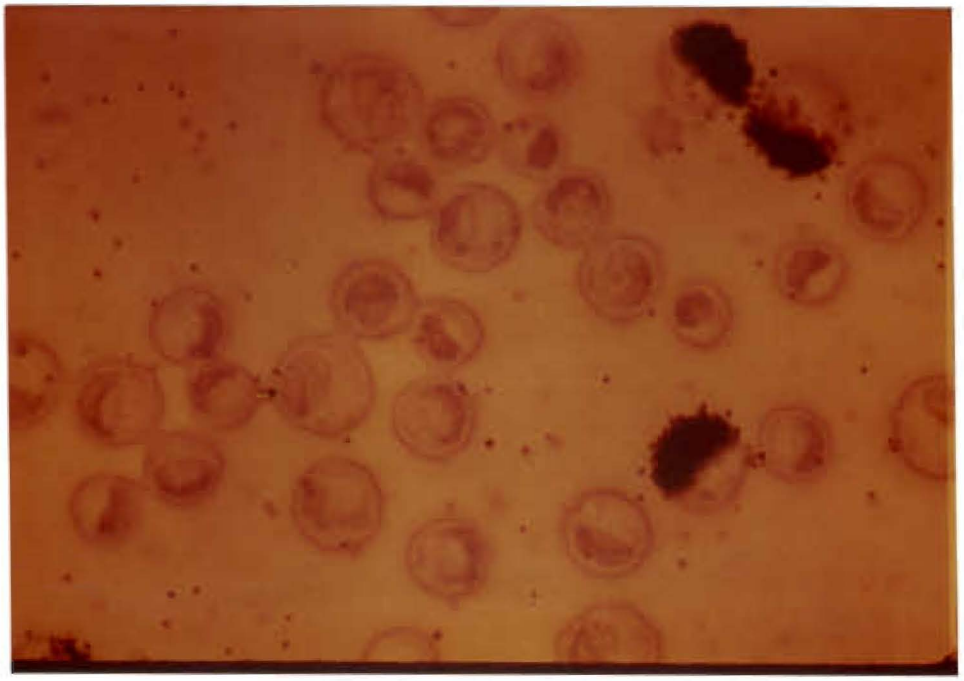
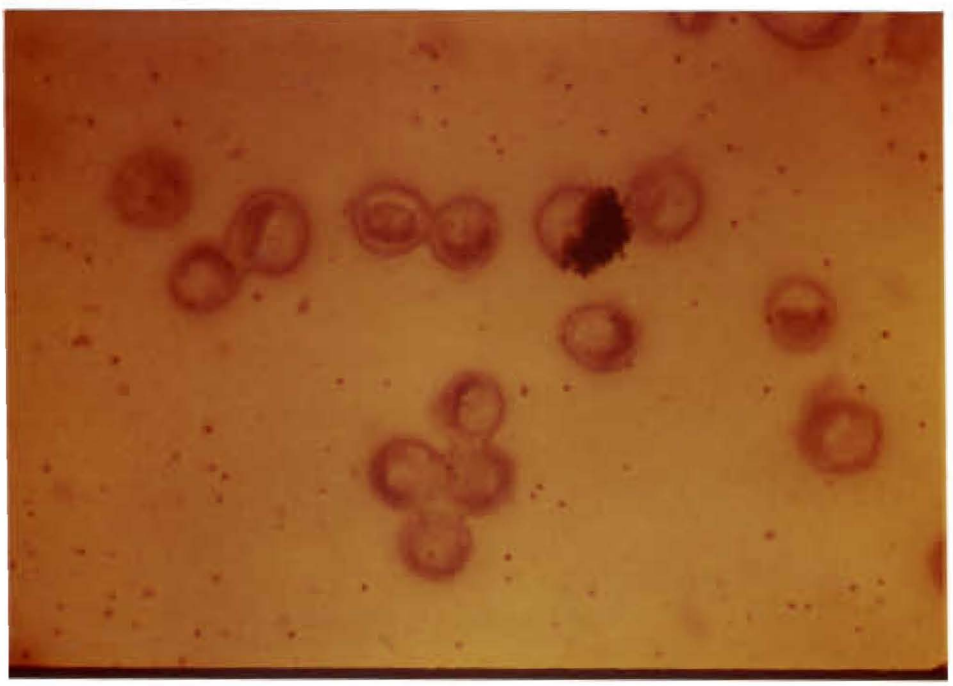


Figure 17. Autoradiograph of normal rabbit PAMs at 8 hours (X 400). No typical cytoplasmic activity noted.

Figure 18. Autoradiograph of immune rabbit PAMs at 8 hours (X 400). No typical cytoplasmic activity noted.





When infected in vitro, pulmonary alveolar macrophages obtained from normal CFA-stimulated and immune CFA-stimulated rabbits supported viral replication in an almost identical manner (Fig. 19). Both cell types adsorbed approximately the same amount of virus, which is demonstrated by the 2 hour post-infection titer. A hundred fold increase in the viral titer at 24 hours was followed by another 100 fold increase at 48 hours. After this time, increase in viral titer in N-PAMs was more gradual, while I-PAMs had a slight decline in viral titer. Both systems, however, showed a 10-fold increase in viral titer between 76 and 96 hours.

Studies of the May-Grunwald Giemsa-stained coverslips at 2 hours indicated that the macrophages were healthy with a slight acidophilic cytoplasm. At this time, a slight tendency for stimulated N-PAMs to aggregate was observed but no sign of cell injury in either type was seen (Fig. 20-21). Cell aggregation of both stimulated cell types was detected in a few foci by 24 hours, and except for a few vacuolated cells, no other signs of cell injury were apparent (Fig. 22-23). Complete cell aggregation without any signs of cell destruction in both stimulated cell types was seen at 48 hours, and in addition, some macrophages on the infected coverslips showed morphological changes (Fig. 24-25). The first evidence of slight cell injury in both stimulated cell types was observed in samples taken at 72 hours (Fig. 26-27). The cell injury did not show any advancement by 96 hours and stayed at the level of the 72 hour samples. At the first stage of infection some binucleated cells were detected, which then transformed into the giant multinucleated cell as the infection progressed.

Figure 19. Time course of viral titer in normal CFA-stimulated (SN-PAM) and immune CFA-stimulated (SI-PAM) macrophages infected with vaccinia.
PFU = plaque forming unit

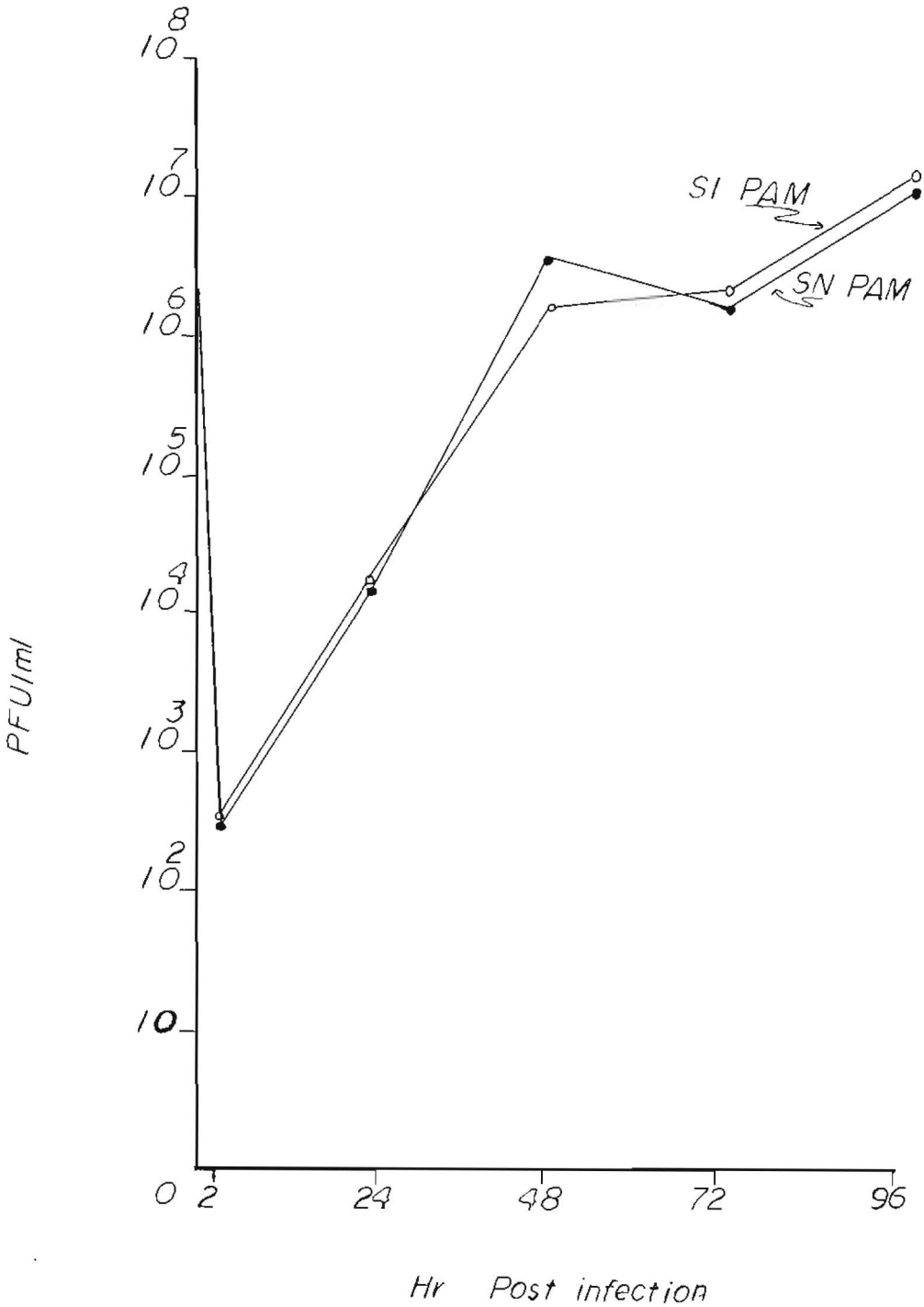
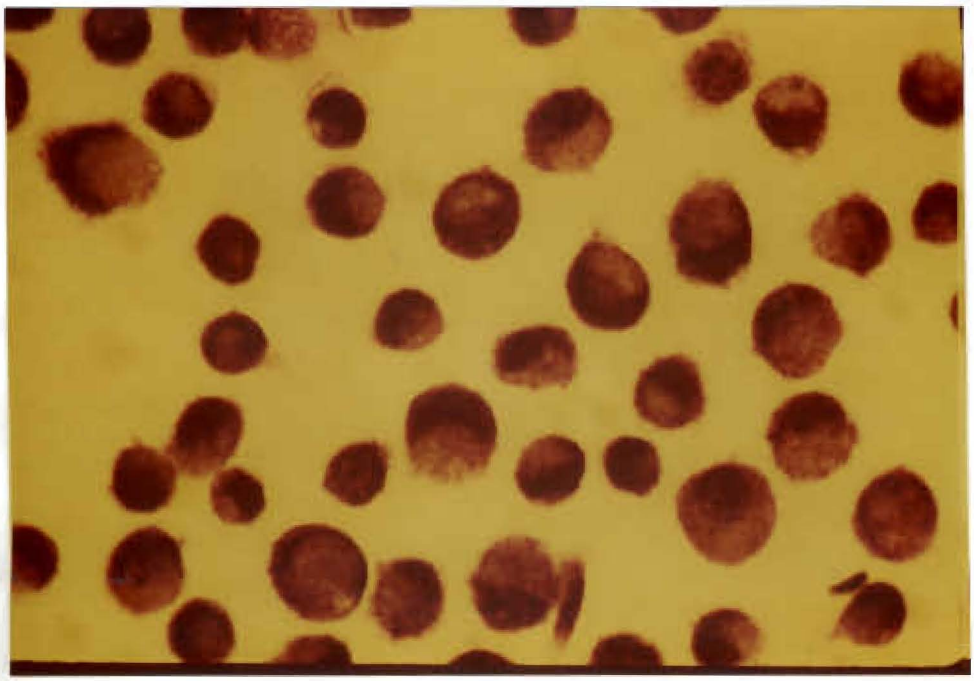

PFU/ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
2
24
48
72
96
SI PAM
SN PAM
Hr Post infection

Figure 20. Normal stimulated rabbit PAMs infected for 2 hours (X 400). No change due to viral infection.

Figure 21. Immune stimulated rabbit PAMs infected for 2 hours (X 400). No change due to viral infection.



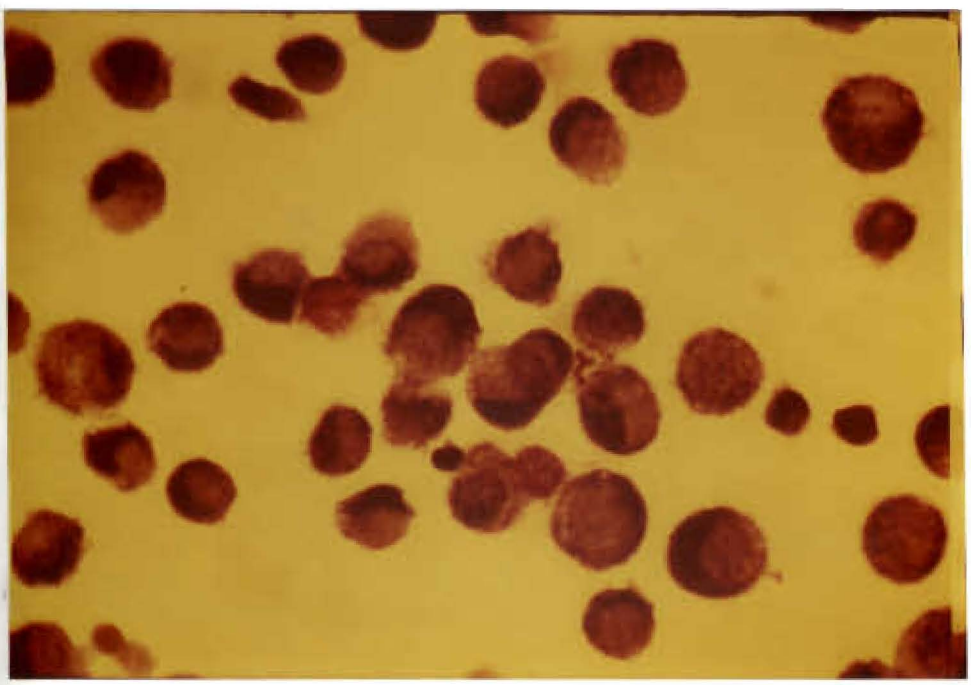
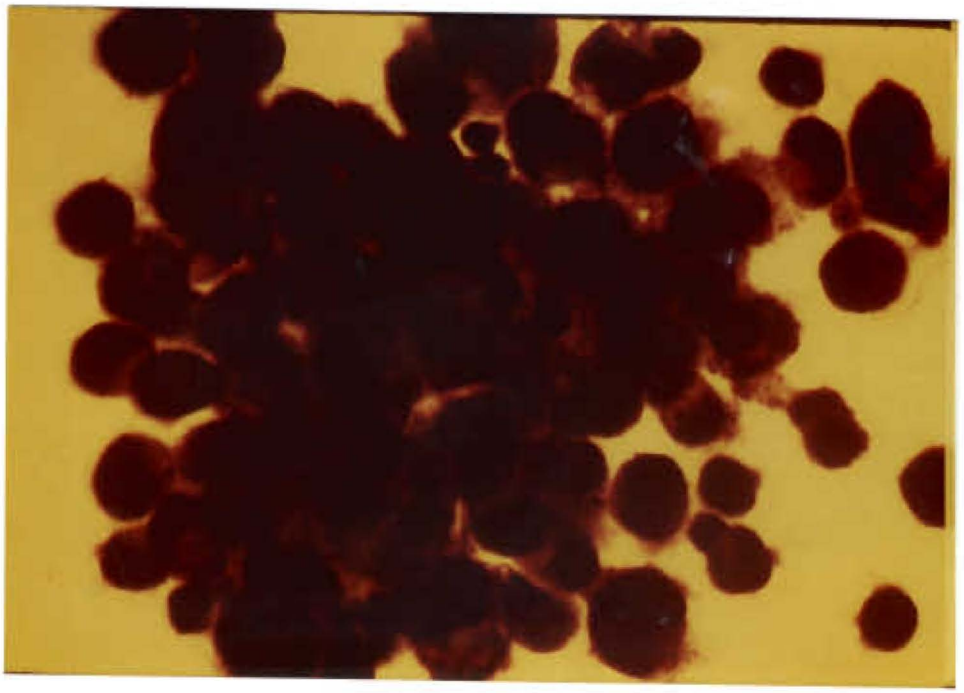


Figure 22. Normal stimulated rabbit PAMs infected for 24 hours (X 400). Showing viral induced cell aggregation.

Figure 23. Immune stimulated rabbit PAMs infected for 24 hours (X 400). Showing viral induced cell aggregation.



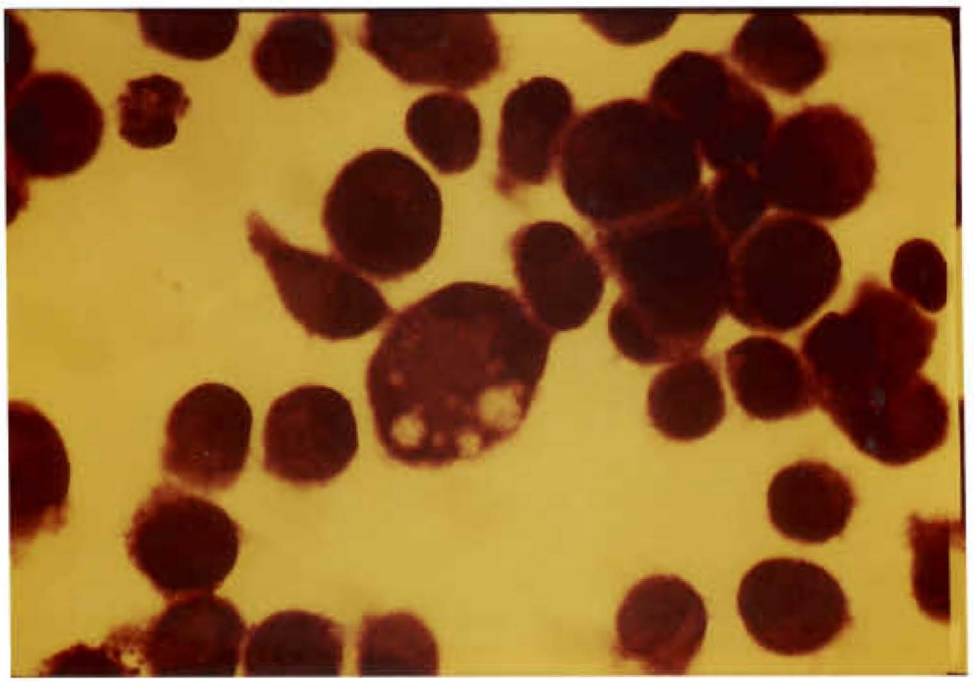
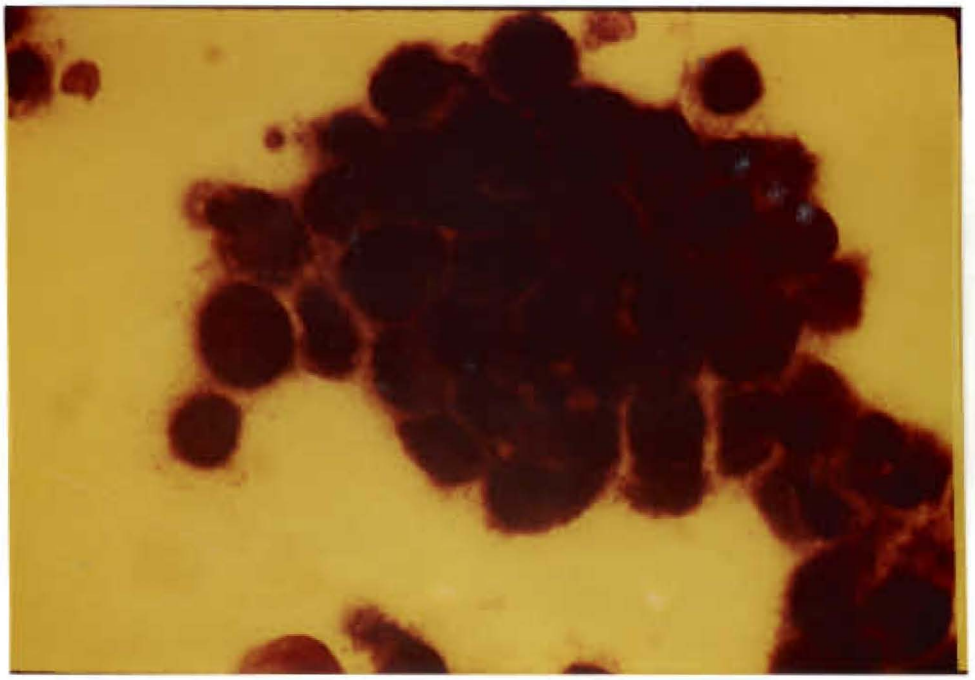


Figure 24. Normal stimulated rabbit PAMs infected for 48 hours (X 400). Note the cell aggregation without cell deterioration.

Figure 25. Immune stimulated rabbit PAMs infected for 48 hours (X 400). Note the cell aggregation without cell deterioration.



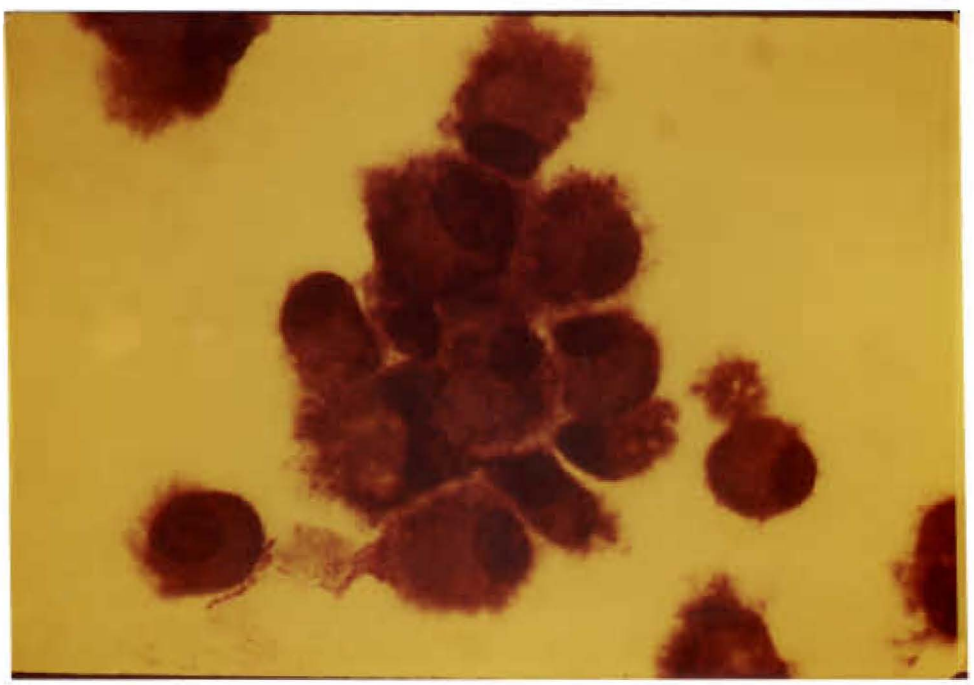
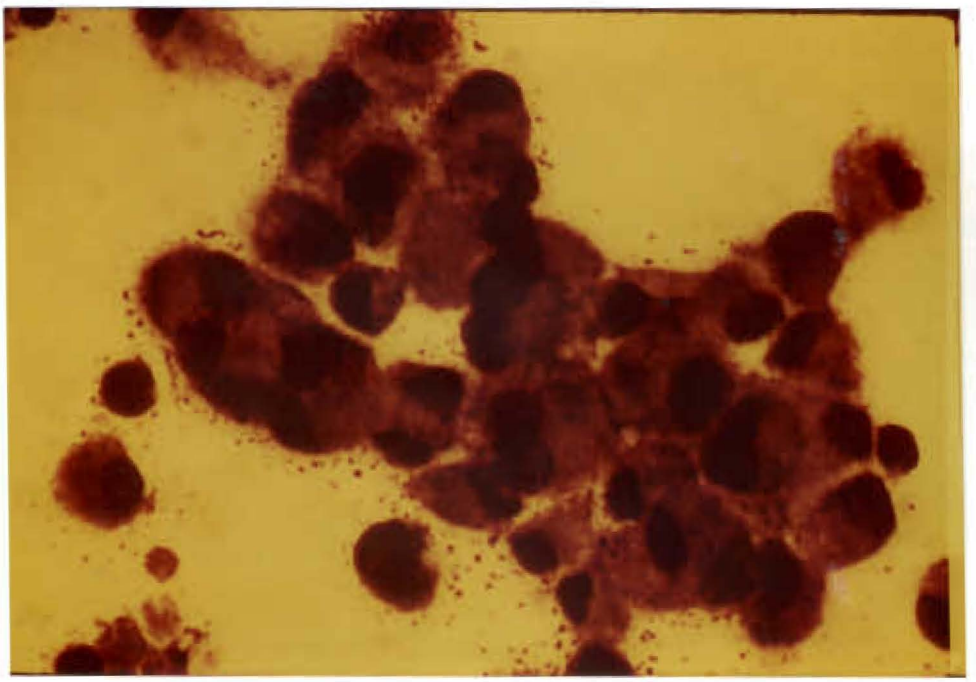
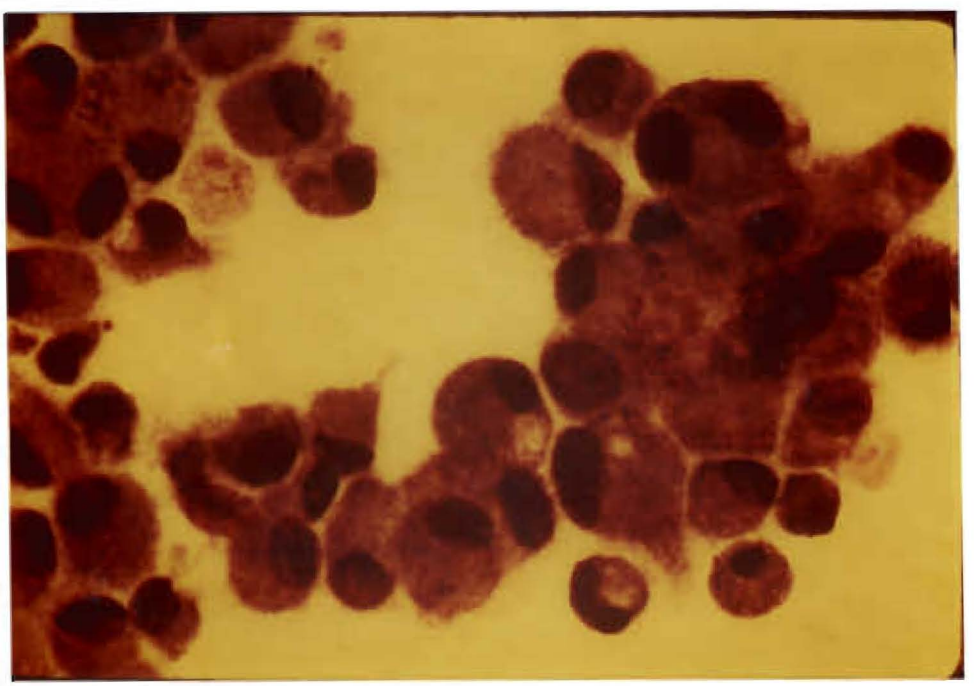


Figure 26. Normal stimulated rabbit PAMs infected for 72 hours (X 400). Cellular cytology remains normal.

Figure 27. Immune stimulated rabbit PAMs infected for 72 hours (X 400). Cellular cytology remain normal.





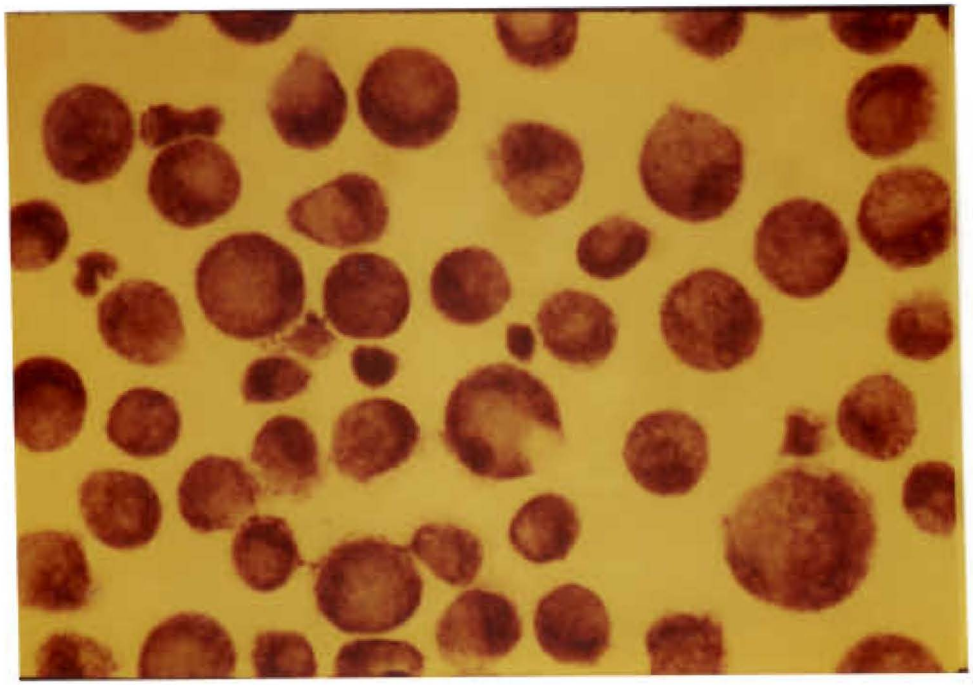
Stimulated control cells, however, were healthy throughout the experiment and no sign of CPE or presence of multinucleated cells was detectable (Fig. 28-29).

Results of the autoradiographic examination of the stimulated PAMs are shown in Figure 30. Samples taken at 8 hours post-inoculation demonstrated 2.4%, 1.8%, and less than 0.2% cytoplasmic activity for stimulated N-PAMs, stimulated I-PAMs, and the control, respectively. About 6-7% nuclear activity was observed but not considered as the site for the viral replication. ^{3}H-TdR incorporation into the viral DNA at replication sites in PAMs' cytoplasm increased to 7.7% for both infected cell types at 24 hours (Fig. 31-32). Marked elevation of the ^{3}H-TdR incorporation was seen at 48 hours and at this time cytoplasmic activity of the stimulated I-PAMs had reached 80% while stimulated N-PAMs showed 55% activity (Fig. 33-34). The cytoplasmic activity of the stimulated N-PAMs stayed at approximately 55% throughout the rest of the experiment, in contrast to the stimulated I-PAMs' activity which was too numerous to count at 72 and 96 hours.

Viral replication was also demonstrated in the mixed culture of of PAMs and PMs, which were infected in vitro. Results are shown in Figure 35. Both immune and normal systems were equally efficient in viral uptake and supported a 10-fold increase by the first 24 hours. At 48 hours the viral titer reached to 10^{6} PFU/ml, about a 100-fold increase over 24 hours. Until this time, both normal and immune system supported the viral replication in an almost identical pattern, but after this time the normal system continued to support the viral replication, whereas the immune system demonstrated a decline in the viral titer by 72 and 96 hours (Fig. 35).

Figure 28. Uninfected normal stimulated rabbit PAMs at 2 hours (X 400). Showing no change due to viral infection.

Figure 29. Uninfected immune stimulated rabbit PAMs at 2 hours (X 400). Showing no change due to viral infection.



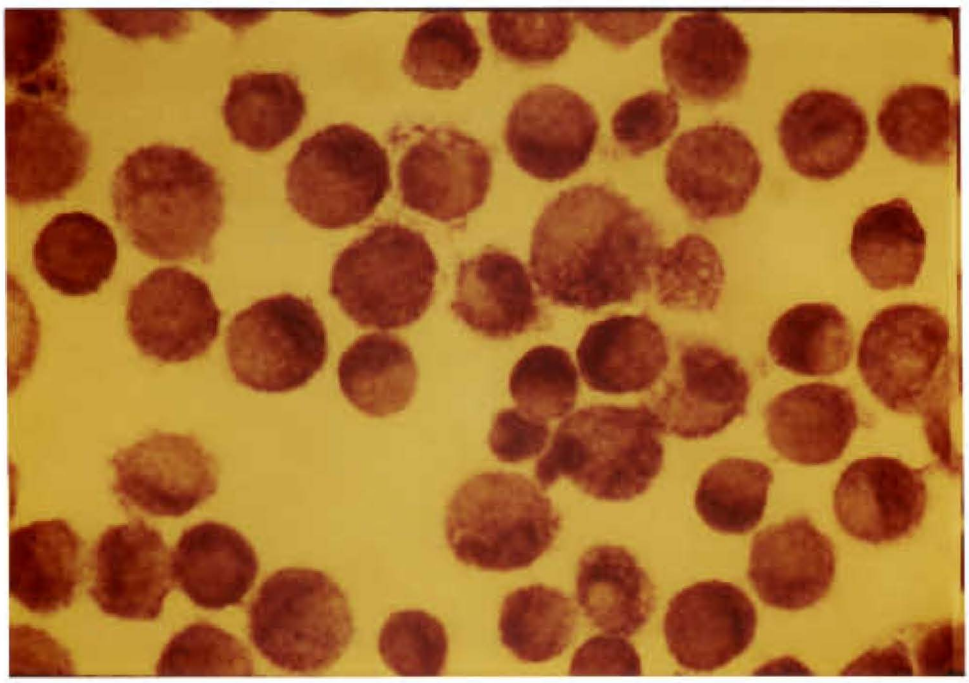


Figure 30. Time course of viral induced DNA synthesis in immune stimulated (SI-PAM) and normal stimulated (SN-PAM) macrophages infected with vaccinia *in vitro*.

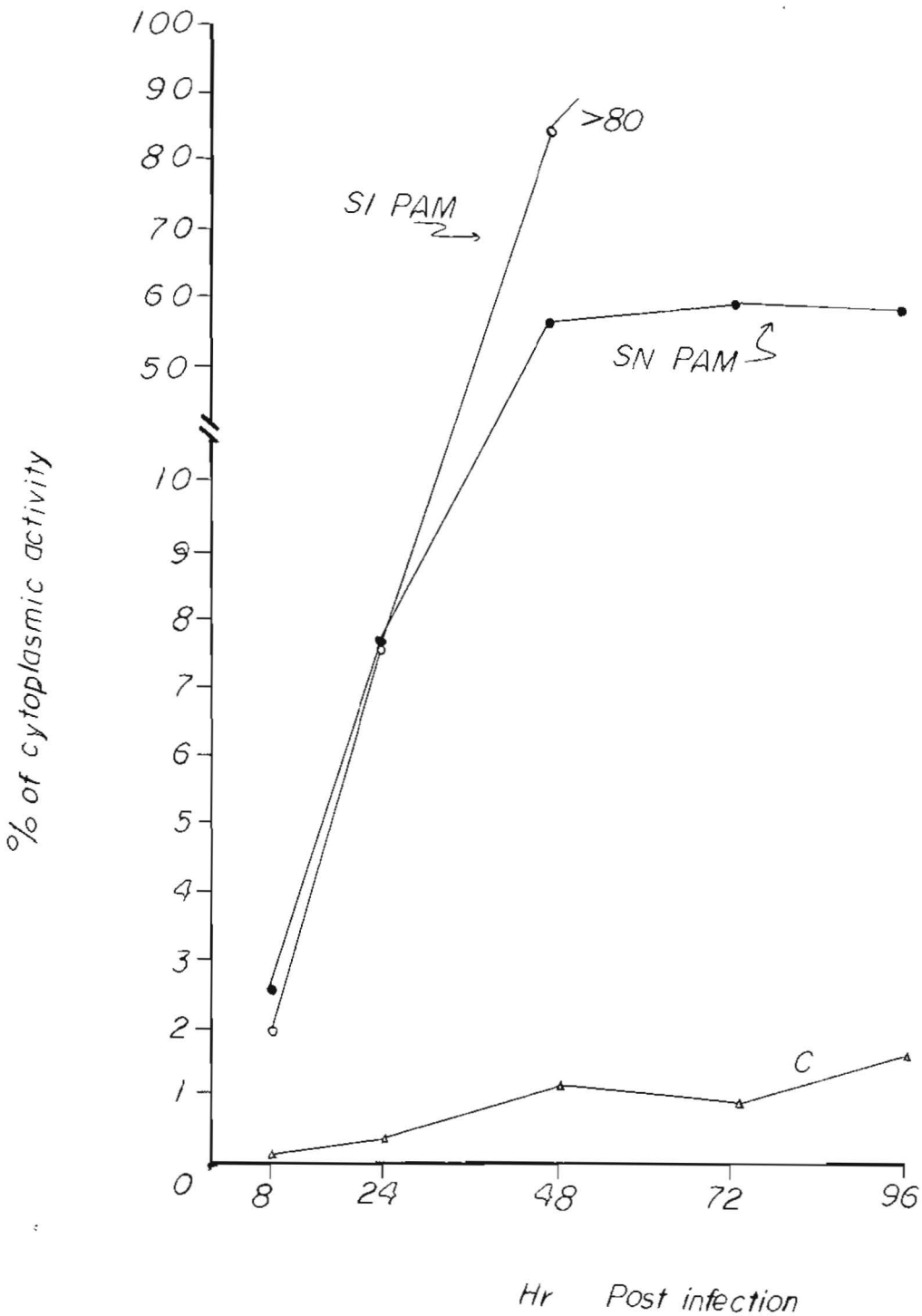
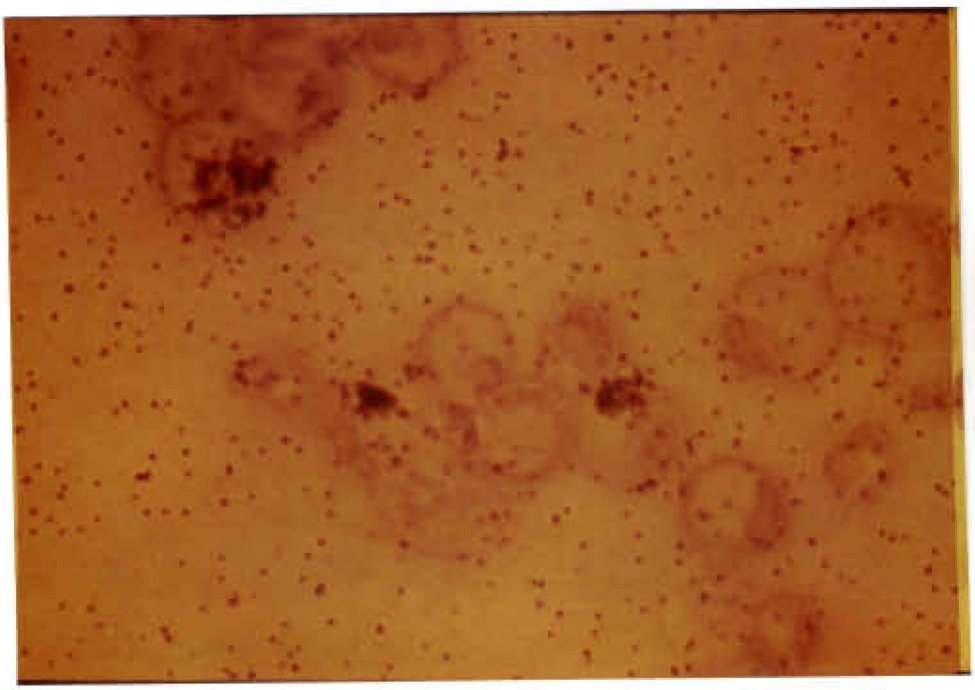

100
90
80
70
60
50
10
9
8
7
6
5
4
3
2
1
0
>80
SI PAM
SN PAM
C
8
24
48
72
96
% of cytoplasmic activity
Hr Post infection

Figure 31. Autoradiograph of normal stimulated rabbit PAMs infected for 24 hours (X 400). Showing viral induced DNA synthesis.

Figure 32. Autoradiograph of immune stimulated rabbit PAMs infected for 24 hours (X 400). Showing viral induced DNA synthesis.



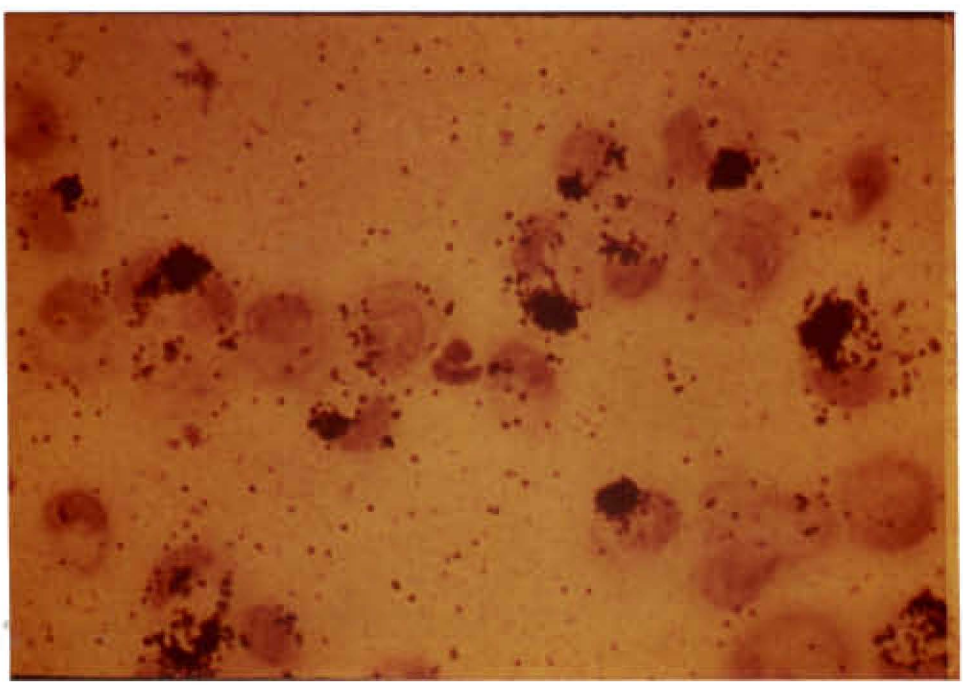
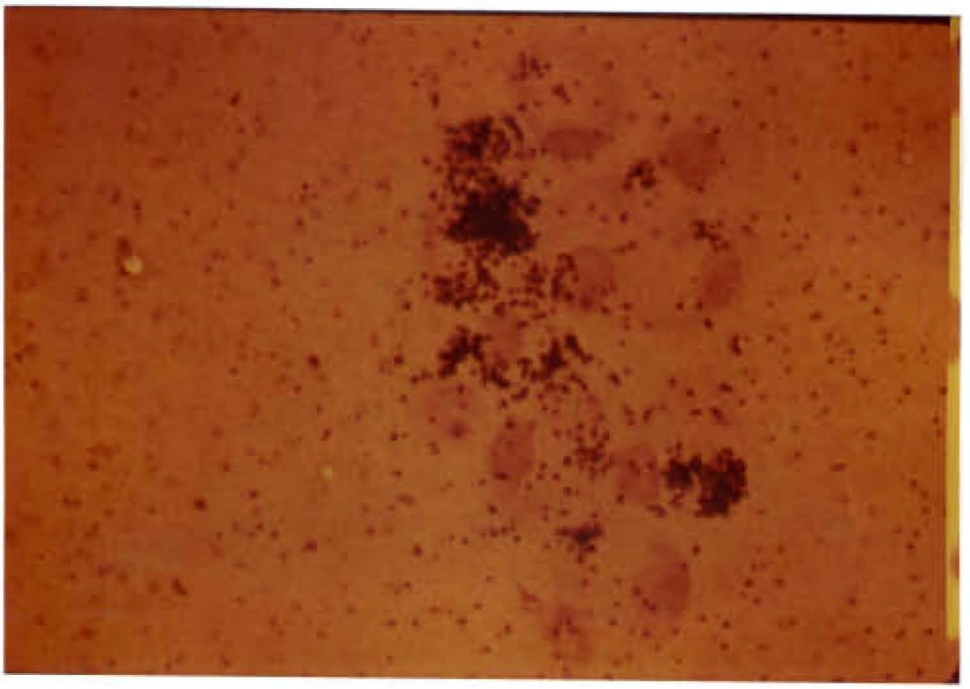


Figure 33. Autoradiograph of normal stimulated rabbit PAMs infected for 48 hours (X 400) with majority of cells exhibiting cytoplasmic activity.

Figure 34. Autoradiograph of immune stimulated rabbit PAMs infected for 48 hours (X 400) with majority of cells exhibiting cytoplasmic activity.



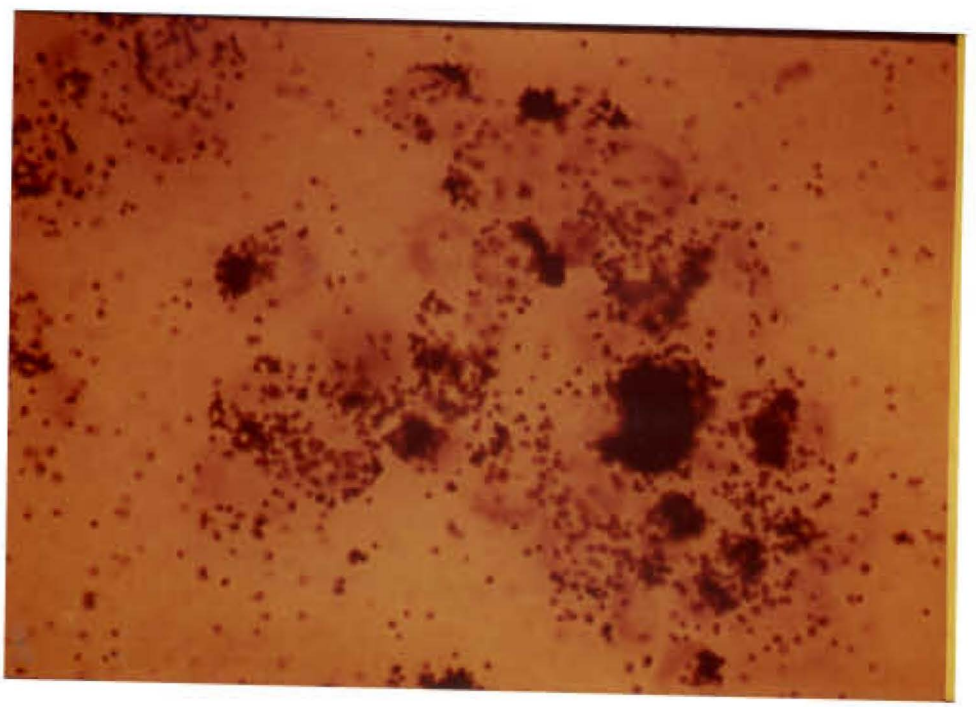
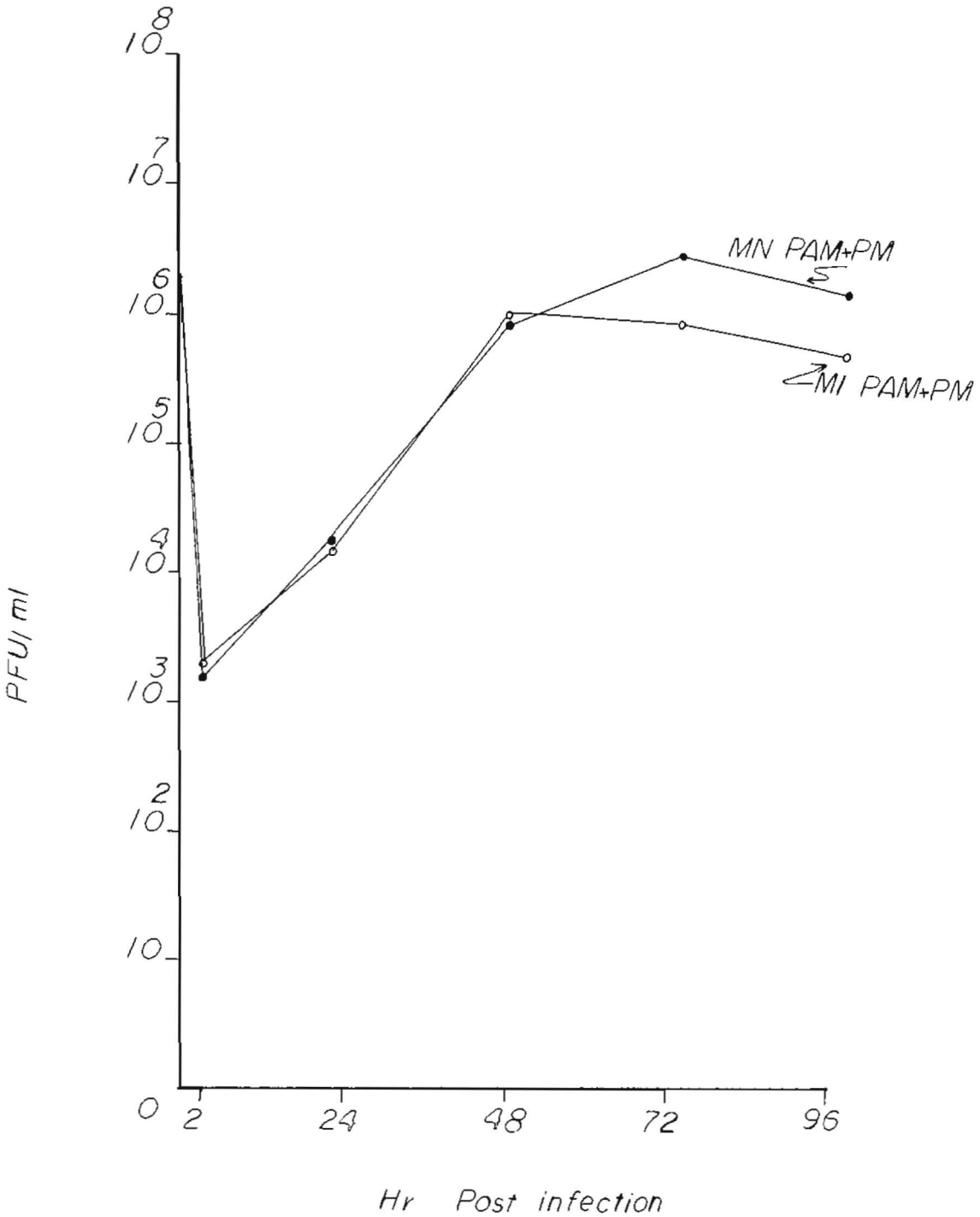


Figure 35. Time course of vaccinia titer in a mixed culture of normal PAMs and PMs as compared to a mixed culture of immune PAMs and PMs.

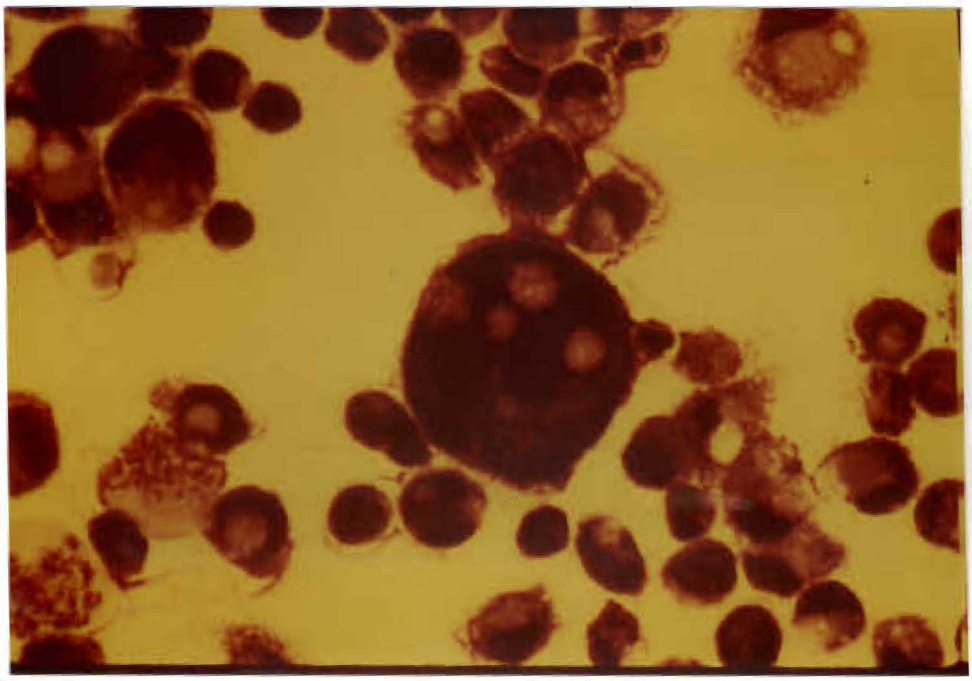

10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
0
2
24
48
72
96
MN PAM+PM
MI PAM+PM
PFU/ml
Hr Post infection

Light microscope examination of these systems at 2 hour post-inoculation revealed some signes of CPE in both infected systems (Fig. 36-37). By 24 hours cell aggregation was evident and slight signs of cell injury were detected. Increased cell aggregation, cell destruction and highly basophilic cells were observed at 24 hours (Fig. 38-39). Cell injury was highly evident by 72 and 96 hours, by which times many cells became detached from coverslips and only a few intact cells were present (Fig. 40-41).

Non-infected control cells manifested a slight acidophilic cytoplasm (Fig. 42-43), which then shifted to slight basophilic staining. Many multinucleated and vacuolated cells were present. The control system showed no sign of cell injury throughout the experiment, but cell detachment and cell loss from coverslips were noticed at 72 and 96 hours.

Figure 36. Mixed normal rabbit PAMs and PMs infected for 2 hours (X 400). Showing a slight tendency to aggregate.

Figure 37. Mixed immune rabbit PAMs and PMs infected for 2 hours (X 400). Showing a slight tendency to aggregate.



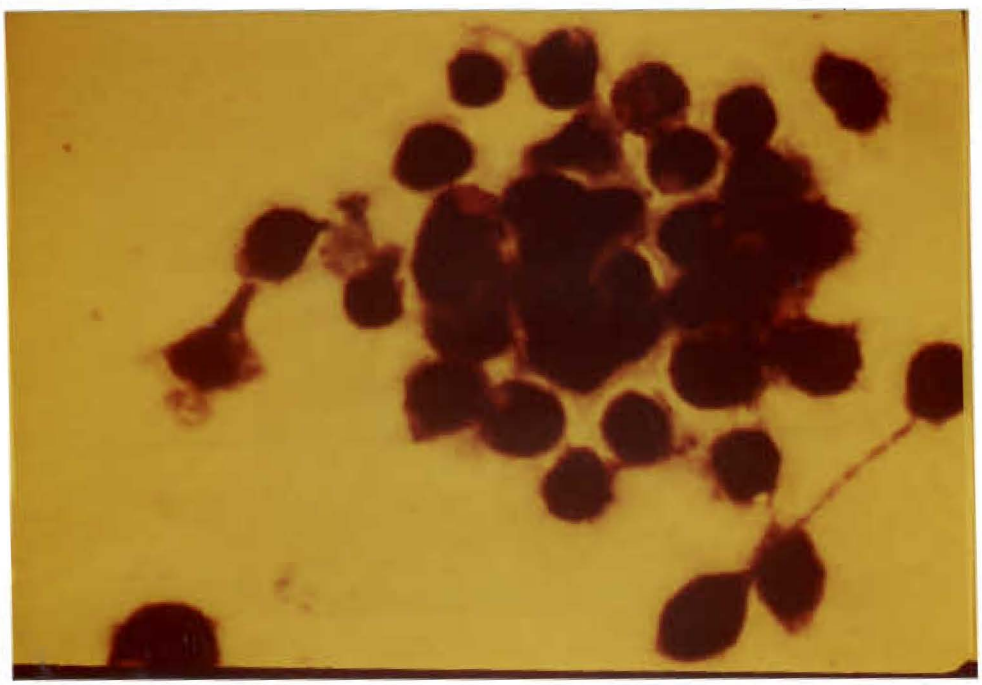
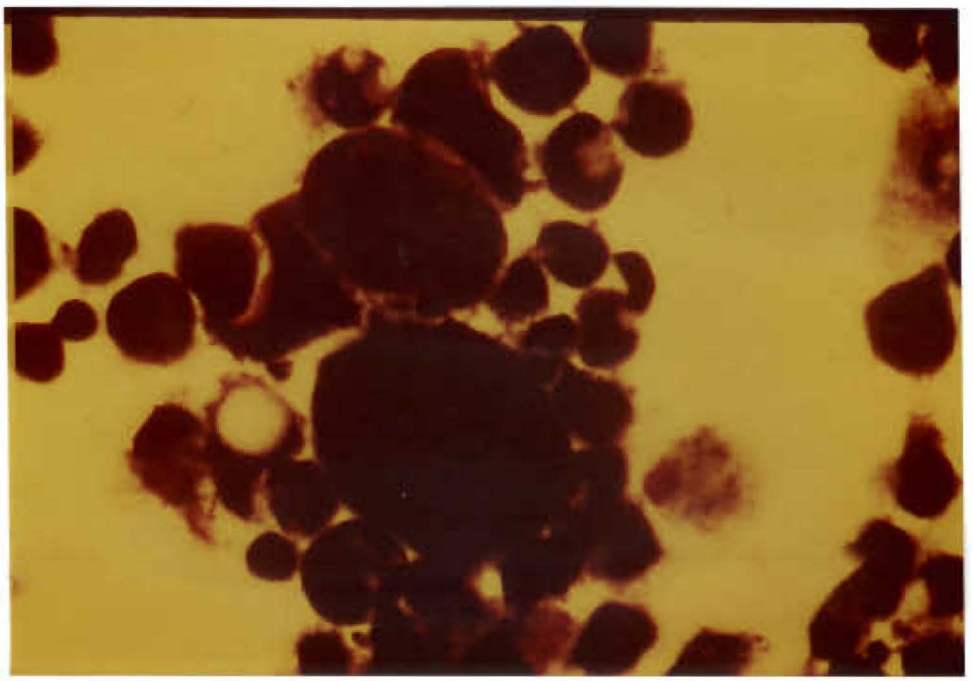


Figure 38. Mixed normal rabbit PAMs and PMs infected for 48 hours (X 400). Showing extensive cell aggregation.

Figure 39. Mixed immune rabbit PAMs and PMs infected for 48 hours (X 400). Also showing cell aggregation.



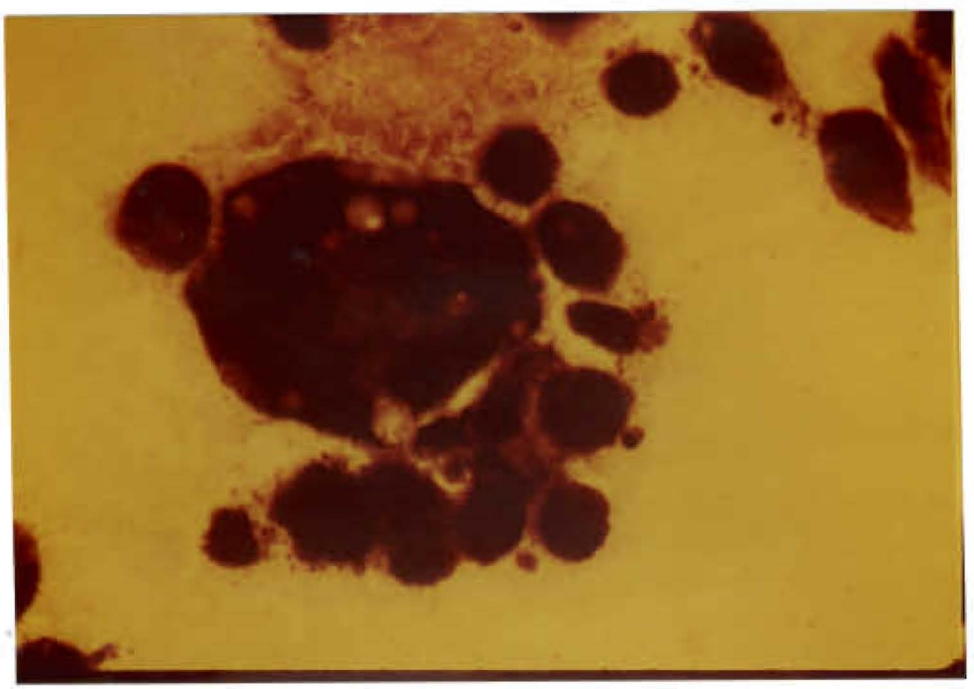
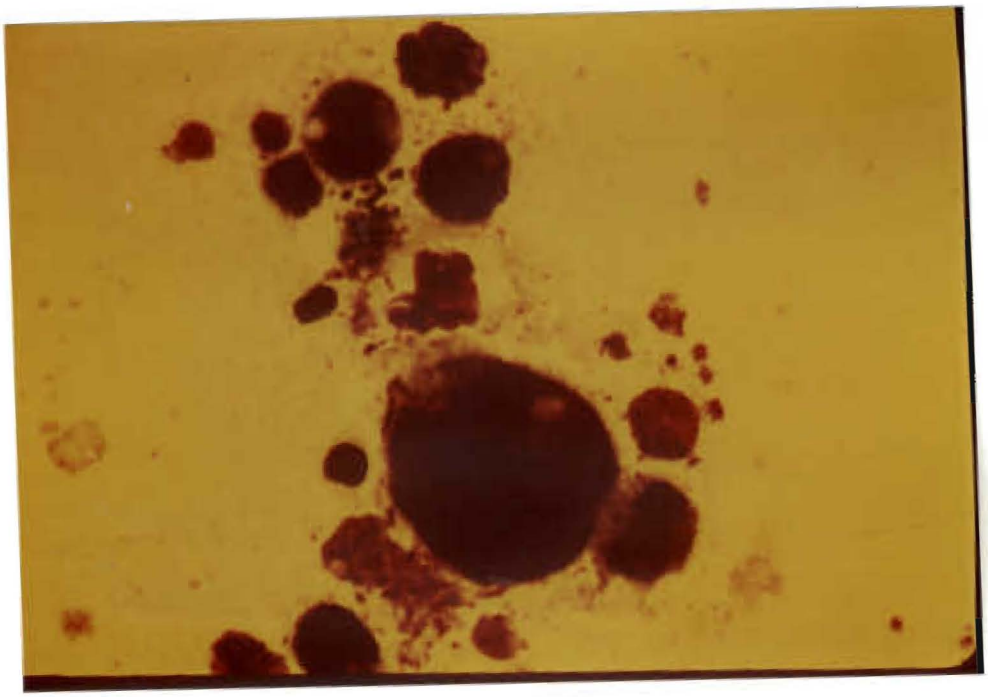
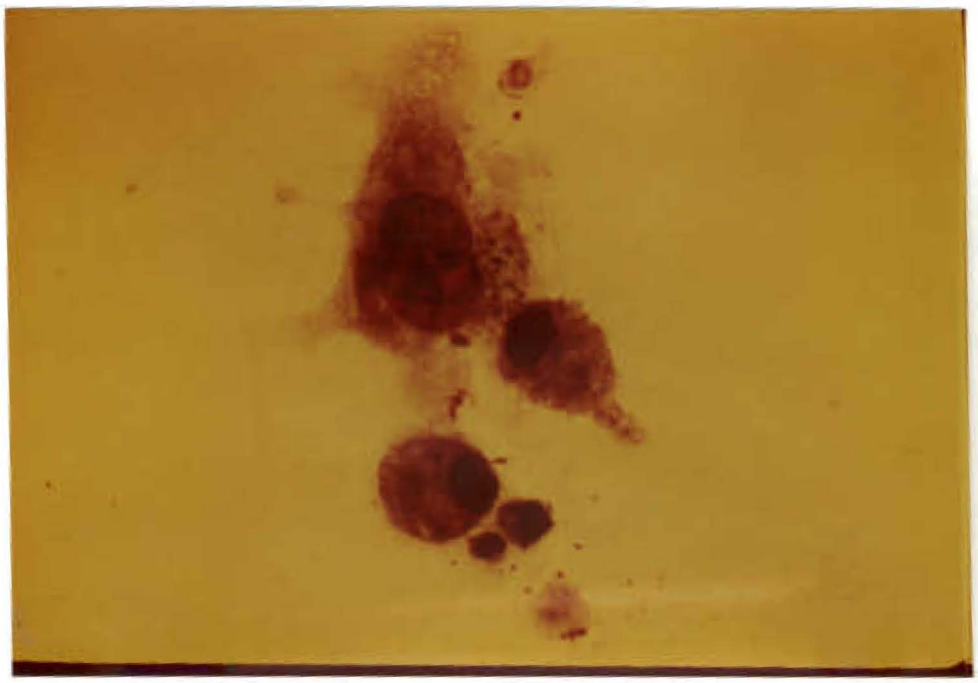
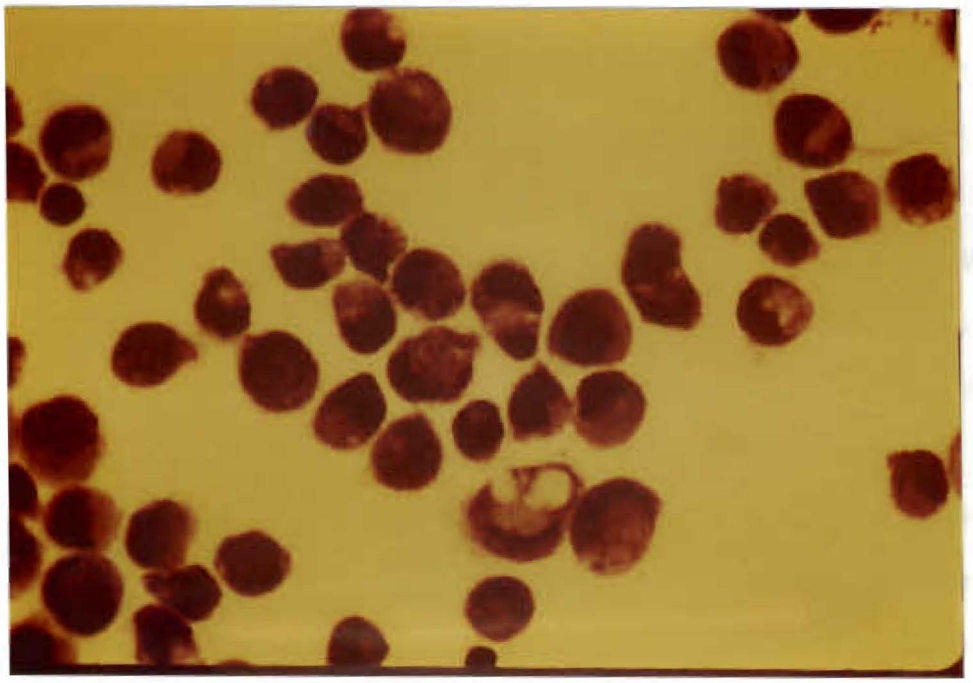


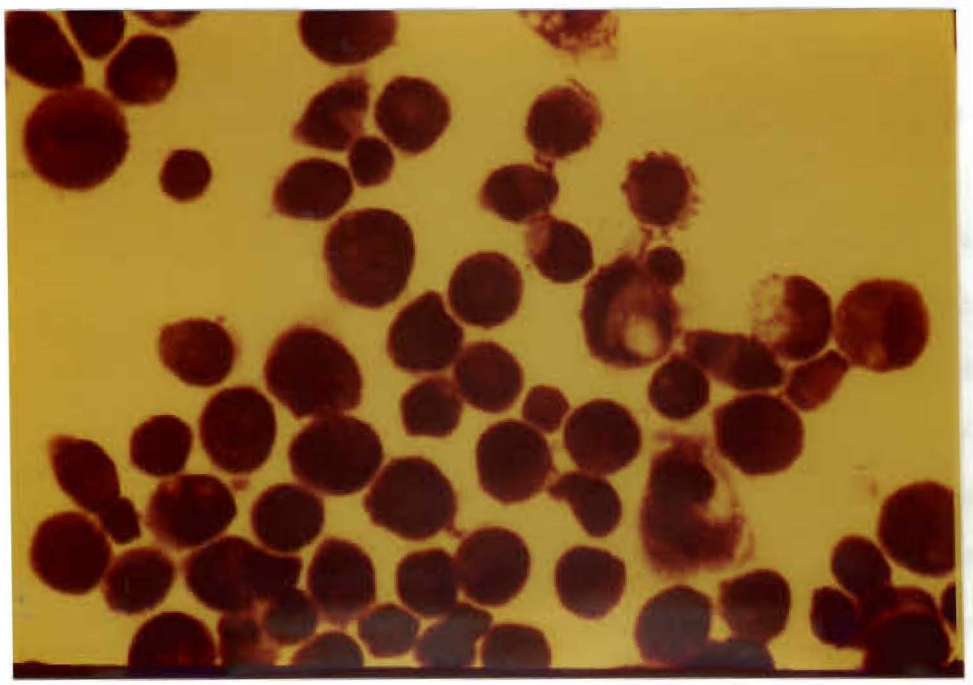
Figure 40. Mixed normal rabbit PAMs and PMs infected for 96 hours (X 400). Note the cell destruction.

Figure 41. Mixed immune rabbit PAMs and PMs infected for 96 hours (X 400). Note the cell destruction.









DISCUSSION

The role of the PAMs, from normal and vaccinia-immune rabbits, in in vitro vaccinia virus infection has been investigated (31, 32). These studies have shown that PAMs, both normal and "immune", were equally susceptible to viral infection and supported viral replication as demonstrated by fluorescent antibody (FA) technique. Using FA, other investigators were able to detect the percentage of the infected PAMs during the course of viral infection. Because FA is sensitive only to the presence of viral antigens, autoradiography was employed in the present study to follow the viral induced DNA synthesis in the cytoplasm of infected cells, thereby measuring a different parameter of infection. With labeled thymidine it was shown that the percentage of PAMs containing typical cytoplasmic areas of activity increased from 3-4% at 8 hours to 30% at 24 hours. At 48 hours labeling exceeded 50%. These data are in accord with the results of those who showed a similar course of viral replication in rabbit PAMs (31, 32). Therefore, it has been well established that vaccinia virus infects and replicates in the cytoplasm of normal and "immune" rabbit PAMs.

However, it is known that physiologically or immunologically stimulated macrophages develop enhanced ability to depress both bacterial and fungal infections (33, 34). It was important, consequently, to investigate the results of infecting stimulated PAMs with vaccinia virus. It was found that there was no significant difference in the pattern of viral reproduction in the stimulated versus the unstimulated PAMs for the first 48 hours. The stimulated PAMs adsorbed $4X10^2$ PFU of virus and viral titer rapidly increased through 48 hours and then more gradually.

The stimulated PAMs did not suppress the viral replication but, in contrast to unstimulated PAMs, they supported viral multiplication without losing their integrity to the same extent as unstimulated PAMs. This fact was demonstrated by comparing the 48, 72 and 96 hours stained samples of each population. It appears that the stimulation of PAMs by complete Freund's adjuvant in some way enhances their resistance to viral injury even though viral replication is taking place. This phenomenon of cell resistance to injury is difficult to explain on the basis of the data collected in the present study. However, it seems clear that this resistance has been acquired during the in vivo activation and its mechanism apparently is related to the enhancement of the physiological activity of PAMs.

Interaction of the vaccinia virus with PMs from normal and vaccinia-immunized rabbits has also been studied (35, 36). These studies showed that the normal PMs were not capable of suppressing the viral infection, while "immune" PMs exerted a suppressive effect on the viral replication by blocking a late step in the virus replication cycle (35). To test the possibility that the PMs might be able to transfer this ability of suppression of viral replication to the PAMs, the two macrophage types were incubated together over-night before in vitro infection of the mixed cultures. By inoculating the mixed cultures with vaccinia virus and monitoring the course of viral activity by titering samples, it was demonstrated that virus was able to grow in the mixed cultures with the efficiency seen in the cultures of unstimulated PAMs. The virus replicated most rapidly through 48 hours, followed by a more gradual increase in viral activity. Damage to cells of the mixed cultures was

as extensive as in the normal PAM cultures. Thus, the co-incubation of the PAMs with PMs did not reveal any effect on the course of viral replication. Rather, the co-incubation appeared to render the PMs as susceptible as PAMs to the vaccinia viral infection.

At present, it is not known why macrophages taken from the lungs of an animal respond to infection differently from macrophages taken from the peritoneal cavity. It is particularly difficult to explain in view of the fact macrophages populating these two anatomical sites originate from the same progenitor cells. However, it seems clear that these two cell populations acquire and express different properties, depending on the particular microenvironment in which they reside.

Very much the same question is raised when PAMs and PMs from an immunized animal are compared in ability to suppress vaccinia replication. The PMs acquire, during the immunization process, an enhanced ability to both suppress vaccinia replication as well as a resistance to injury from viral infection (36, 36). In marked contrast the CFA-stimulated PAMs from the vaccinia immunized animal failed to inhibit viral reproduction although they are more resistant to viral cytopathic effects than their unstimulated counterparts.

Again, the differences between PAMs and PMs may be a reflection of regional environmental differences that affect the immune potential of these populations in distinct ways. In a review article Walker (37) has discussed some of the differences between PAMs and PMs. These differences are reflected in bactericidal and Fc receptor activity, which PMs possess in much higher degree than PAMs. He also indicated three other properties that PAMs lack: ability to process ingested

antigens to stimulate immunological response, chemotaxis and formation of immunogenic RNA's. Because of these differences, it was surprising to find that the normal PAMs and normal PMs are similar in response to vaccinia viral infection, with both supporting viral reproduction (32, 36). Therefore, it can be suggested that both cell types acquire resistance to the vaccinia viral infection during the immunization, but in time PAMs lose or exhaust their acquired properties during the constant contact with foreign agents, while PMs rarely have any chance to contact them in vivo.

The present study raises the question of exactly what role the PAMs assume in the protection of the host against viral infection. Obviously, effective protective mechanisms must be present in the lungs of a host because the number of virus species with the potential to produce respiratory diseases is very large. It has been shown that antibodies play a crucial role in the lungs (15), as do thymus-derived lymphocytes (38, 39, 40), but it would be a rather unexpected finding if macrophages also did not have a part in respiratory immunity. However, the protective function of these macrophages, particularly against POx viruses, has yet to be defined.

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LITERATURE CITED

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