THE EFFECT OF PHYTOHEMAGGLUTININ ON VACCINIA REPLICATION *IN VITRO*

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INTRODUCTION

In the last few years phytohemagglutinin (PHA), an extract derived from red kidney beans (*Phaseolus vulgaris*), has been the subject of numerous studies because of its ability to stimulate DNA synthesis in certain cells and to agglutinate both erythrocytes and leucocytes in a wide variety of animals. In addition, PHA has recently been shown to allow the replication of certain viruses in cells which would not otherwise be susceptible to the virus (Nahmias *et al.*, 1964; Duc-Nguyen and Henle, 1966; Wheelock, 1965; Miller and Enders, 1968) and to increase the replication of some viruses in susceptible cells (Edelman and Wheelock, 1968).

Little is known about the chemical composition of PHA and its mode of action in agglutinating cells and initiating mitosis. It is believed that PHA is a macromolecule, extractable in both a mucoprotein and a protein form (Rigas and Osgood, 1955). It has been used as a basic tool in cytogenetics for chromosomal analysis since Hungerford *et al*, (1959) first described its mitogenic effect in lymphocyte cell cultures. It has been reported that PHA induces cell transformation to blast-like cells capable of undergoing mitosis in all forms of lymphocytes, with recent evidence suggesting that only thymus derived lymphocytes of lymphoid cell populations are stimulated to transform by PHA (Doenhoff *et al.*, 1970). PHA's mode of action and localization in leucocytes has been a subject of much controversy since its leucocyte associated

properties were first described. These properties are leucoagglutination (Hastings et al., 1961), stimulater of RNA synthesis (Rivera and Mueller, 1966), and an inducer of DNA synthesis (Nowell, 1960).

There has been little work with the effect of PHA on cells other than lymphocytes. Smith and Goldman (1971) investigated the PHA response to certain types of macrophage cultures and observed that the PHA interaction with human colostral macrophages was distinct from the interaction with mouse peritoneal macrophages. In contrast to lymphoid cell cultures, blast cells were not observed in the macrophage cultures.

The ability of PHA to stimulate DNA synthesis of certain cells prompted an investigation of PHA's effect on a DNA virus (vaccinia virus) infection in susceptible host cells. Although vaccinia virus replication takes place in the cytoplasm of its host cell and does not depend on DNA synthesis in the host nucleus (Dales, 1963), Miller and Enders (1968) reported that vaccinia replication occurred in a previously non-susceptible host cell, the human peripheral blood leucocyte, only in the presence of PHA.

This report investigates the effects of PHA on vaccinia replication and ingestion in rabbit peritoneal mononuclear phagocytes. Peritoneal phagocytic cells were used as susceptible host cells because of: (1) their ability to facilitate vaccinia replication, (2) their specific role in host protection against infection via their highly evolved phagocytic properties, (3) the lack of information on the phytomitogen-macrophage interaction.

HISTORICAL BACKGROUND

General

Substances with *in vitro* hemagglutinating activity have been obtained from a variety of plants. As early as 1897 a hemagglutinin was obtained from the castor bean and used by Ehrlich (1897) in his first *in vitro* studies of the reactions between antigen and antibody. Landsteiner (1936) discovered the hemagglutinating property of a substance from red kidney beans which he called phytohemagglutinin (PHA). In 1909 Wienhauns (1909) obtained partially purified protein from red kidney beans with hemagglutinating activity and speculated that the protein was an albumin. It wasn't until 1955 that Rigas and Osgood (1955) described a method for purification of the hemagglutinin obtained from kidney beans, in which they separated both a mucoprotein and a protein. They found that the phytohemagglutinin in either the mucoprotein or the protein form was a non-toxic, powerful hemagglutinin of human erythrocytes, and those of the horse, pig, dog, cat, rabbit, chicken, and frog.

Dorset and Henly (1916) used PHA to remove red cells in the preparation of antiserum against hog cholera. Thirteen years later Goddard and Mendel (1929) described a method for the preparation of PHA, injected it into rabbits and mice and found it not toxic *in vivo*.

After the earlier findings, no further work with PHA was reported until Li and Osgood (1949) utilized it to obtain suspensions of

leucocytes free of red blood cells. Osgood and Krippaehne (1955) used the same procedure in tissue culture work to eliminate mature erythrocytes from their cell cultures.

Probably the most significant finding connected with PHA research came when Hungerford *et al*. (1959) described its mitogenic and blastogenic properties. They found that PHA induced transformation of small lymphocytes, in human peripheral blood, into large primitive cells capable of undergoing division. As a large number of cells in mitosis could be obtained by incubating peripheral leucocytes with PHA, this discovery was of great importance, especially in the field of cytogenetics, by providing a simple and reproducible method for chromosomal analysis.

Although Hungerford *et al*. (1959) were the first to report the mitogenic effects of PHA, their primary interest was using it as a tool in chromosome analysis. Nowell (1960), however, was the first to study its effect on lymphocytes as a mitotic stimulater. He theorized that the action of PHA on leucocytes may be similar to its action on erythrocytes in which there is an agglutinating effect resulting in the linkage of the euglobulin portion of the PHA molecule with a polysaccharide on the red cell surface. From his observations, he introduced the first of several theories concerning the action of PHA in leucocyte cell cultures. His theory implied that PHA may alter the cell membrane to permit entrance of some substance from the culture medium, which in turn initiates the mitotic processes. This substance, however, could not initiate mitosis in the absence of PHA. It was suggested that PHA's action on leucocytes was not to stimulate mitosis *per se* but rather to initiate

the conversion of monocytes and certain lymphocytes to a state capable of division.

The findings that PHA had the remarkable property of inducing blastogenesis and mitosis when incubated with human peripheral leucocytes stimulated further attempts to separate the various active constituents of PHA and to better characterize its chemical structure. Punnett, Punnett, and Kaufmann (1962) reported the separation of two active constituents obtained from cotyledons of *Phaseolus vulgaris*, one capable of agglutinating erythrocytes and the other capable of inducing blastogenesis in tissue culture.

Barkhan and Ballas (1963) showed that the agglutinating activity of PHA could be removed by absorption with red blood cells without a concomitant loss of mitogenic activity, supporting the view that the mitogenic and hemagglutinating activities are expressed by different entities. However, when they attempted to separate the mitogenic and hemagglutinating principles by starch block electrophoresis, they could only recover the hemagglutinating activity. Presumably, the mitogenic activity was destroyed during the fractionation procedure. Removal of the erythrocyte agglutinating activity of PHA was also achieved by Robbins (1963). He incubated PHA with packed red cells at 4 C for 25 minutes, centrifuged the cell suspension and repeated the absorption a second time. He found that the supernatant solution did not possess erythrocyte agglutinating activity but had retained essentially all of the blastogenic and mitogenic activity of the original preparation.

Hastings *et al.* (1961) observed that PHA also agglutinated blood leucocytes and that mitosis of PHA treated leucocytes occurred only in

white blood cell clumps, suggesting that the leucoagglutination could be partially responsible for the mitotic activity. Kolodny and Hirschhorn (1964) separated the erythroagglutinating property of PHA by the red blood cell absorption technique and found that the supernatant was not only capable of inducing mitosis but that it would also agglutinate leucocytes. However, when PHA was then absorbed with white blood cells, all three activities were removed. The authors concluded that there was an apparent relationship between the mitotic and white blood cell clumping activities.

After it was established that·the leucoagglutinating and mitogenic activity of PHA were distinct from its erythroagglutinating property, Rivera and Mueller (1966) presented evidence for the existence of at least three separate and distinct activities of PHA affecting lymphocytes; a leucoagglutinin, a factor inducing RNA synthesis, and a factor inducing DNA replication.

Barberg et al. (1966) found that N-acetyl-d-galactosamine (5 mg/ml) selectively inhibits the agglutination of rat thoracic-duct lymphocytes by PHA and that cells freshly agglutinated by PHA could be disassociated by the addition of the sugar at a ten-fold concentration (50 mg/ml). The blastogenic and mitogenic activities of PHA were not affected. The sugar was also shown to selectively inhibit agglutination by PHA of rat thymus, rabbit thymus, and human tonsil cells, and also human, rat, and rabbit erythrocytes. Their results indicated that the inhibitory effect of N-acetyl-d-galactosamine was due neither to the destruction of PHA nor to an irreversible alteration of the cell. They suggested that the sugar acts in a manner analogous to a hapten

participating in the interaction as a constituent of the cell surface, since it has been shown that N-acetyl-d-galactosamine occurs as an external accessible constituent of mammalian cells.

Research with phytohemagglutinin has developed into such a wide field of the bio-sciences, that it is beyond the scope of this paper to describe all of its properties and developments. Although the research has intensified greatly in the last few years, there is still relatively little known about the PHA-leucocyte interaction. The pertinent properties of PHA dealt with in this paper are its mode of action and localization in monocyte cell cultures. It should be emphasized that the majority of the immunological research with PHA has been limited to lymphocyte cell systems, therefore, the properties described are not necessarily valid for all other monocytes.

Localization of PHA

Michalowski *et al*. (1964) studied the cellular localization of the mitogenic principle of PHA in human peripheral leucocytes by labeling PHA with fluorescein isothiocyanate (FITC). During the early period of culture (4-9 hrs) a distinct localization of fluorescent material was found confined to the nuclei of the majority of the lymphocytes, but not the granulocytes. This localization of fluorescein-conjugated PHA did not change during the entire three day culture. The authors suggested that the labeled mitogenic factor enters the cell and resides inside the nucleus. Three years later, Razavi (1967), also using fluorescein-conjugated PHA, observed fluorescence in white cells of all types during the initial period of culture. Subsequently, the fluorescence localized in the cytoplasm of the blast cells and was not

associated with nuclear structures or the cytoplasmic membrane. In a later paper, Michalowski et al. (1965) used fluorescein conjugated anti-PHA serum and found the localization of the tagged protein within mononuclear cells as first being found on the cell membranes and later in the cytoplasm. Nuclear localization of PHA was not observed in the cells, nor was it found on chromosomes of dividing cells as reported earlier (Michalowski et al., 1964). A decrease in the amount of PHA observed on the 2nd and 3rd day cultures was explained as intracellular digestion of the PHA molecules.

Conrad (1967) obtained tritium-labeled PHA by growing the red kidney bean plants in a nutrient solution containing tritiated water. The extracted PHA was then added to cultures of human blood leucocytes and the results showed that most leucocytes were labeled after 4-6 hours. At first, the label appeared to be mainly in the cytoplasm, but after 2-3 days the nuclear labeling became more intense and was predominant in the nuclei of the large blast cells.

More recently, Berman and Andrews (1970) determined from human peripheral blood cultures that the blastogenic and hemagglutinating properties of PHA were not destroyed by conjugation with fluorescein isothiocyanate and fluorescence from conjugated PHA was seen in the cytoplasm of blood macrophages and on platelets. Although blastogenesis occurred, they found no fluorescence in lymphocytes, altered lymphocytes or blast cells as the previous authors had reported (Michalowski et al., 1964, and Razavi, 1966). In attempting to explain this discrepancy, the authors suggested that conjugation may have denatured a portion of the PHA protein which in turn could have been ingested by the macrophages or

platelets, while a separate, unlabeled fraction or subunit directly initiated blastogenesis.

Mode of Action of PHA

After Nowell (1960) proposed his theory of PHA altering the cell membrane to permit the entrance of some substance which in turn initiated mitosis, other investigators began searching for the mode of action of PHA in leucocyte cultures.

Cooper, Barkhan, and Hale (1963) suggested that PHA may be producing its mitogenic effect by permitting the induction and progress of DNA synthesis. Elves and Wilkinson (1962) described similar evidence when they found that PHA caused a rejuvination of the intermediate lymphocytes, causing a general increase in metabolism particularly in respect to increased DNA synthesis.

Hastings *et al*. (1961) observed that mitosis occurred only in white blood cell clumps, indicating that leucoagglutination may be partially responsible for the increased mitotic activity. Kolodny and Hirschhorn (1964) also supported this view after they discovered that when leucoagglutinating activity was removed by repeated absorptions, the mitogenic activity was lost. Their results indicated that either the mitogenic activity is dependent on leucoagglutination or the two properties were related to a common mechanism, such as binding of PHA to the lymphocyte with a resulting alteration of the cell membrane.

Pearmain, Lycett, and Fitzgerald (1963) suggested a specific immunological response to PHA, which acts as a specific antigen. They suggested that since PHA agglutinated red and white blood cells, and indiscriminately stimulates blood lymphocytes cultured *in vitro* to

undergo blastic transformation, PHA might be considered a universal antigen. Holland and Holland (1965) reported that PHA formed a precipitate indiscriminately with serum. They found, however, that PHA also formed precipitins with pure bovine serum albumin and fractions of human serum containing no immune globulins, in which case it is unlikely that PHA acts as an antigen in precipitin reactions.

Allan, Auger, and Crumpton (1971) observed that neutralization of the lymphocyte stimulating activity of PHA by purified plasma membranes of pig lymphocytes was in disagreement with previous suggestions that stimulation is mediated by attachment to glass (Forsdyke, 1968), by interaction with a serum component (Forsdyke, 1967), or by interaction with cells other than lymphocytes (Levis and Robbins, 1970). Their results were in agreement with Nowell's (1960) original theory that lymphocyte stimulation is mediated by the surface of the cell, and that the initial event in transformation is the interaction of the mitogenic principle with a receptor on the cell surface.

Lindahl-Kiessling and Peterson (1969a) investigated the nature of the binding of PHA to lymphocytes by exposing the lymphocytes to different enzymes before cultivation with PHA. They found that trypsin, pronase, and Vibrio cholera extract suppressed the PHA response, but gradually, after enzyme digestion, the lymphocytes recovered their response potential. This implied that a regeneration of the surface had to occur before the PHA response could be initiated. Sialic acid components of the cell surface were found to be involved, possibly as the target for enzyme action, in the initial stage of the PHA stimulation. In a later study they proposed that enzyme treatment destroyed or

removed the sialic acid containing compound on the cell surface and that it had to be replaced by synthesis or uptake from the medium before PHA could again be utilized (Lindahl-Kiessling and Peterson, 1969b).

Lindahl-Kiessling and Mattsson (1971) showed that a reaction between PHA and cell receptors, which does not include active transport of PHA into the cell, is sufficient to trigger an increased mitotic activity in the lymphocytes. PHA was thought to induce the chain of events leading to mitotic transformation while still on the cell surface or otherwise easily available. They observed that PHA was taken up irreversibly by the cells, without any reactions taking place requiring energy from glycolysis or the citric acid cycle. This suggested that PHA is not taken up actively, as endocytosis is considered to be prevented completely by inhibition of the energy generating systems. Further observations revealed that treated cells can propagate the stimulatory effect to other autologous cells, even though very little free PHA can be detected in the media, indicating that active PHA is still present on the cell surface and can bind with other cells.

In his most recent work, Lindahl-Kiessling (1972) described comparison studies between PHA and concanavalin A (Sumner and Howell, 1936), which supported the theory that mitogens are primarily associated with changes they induce in cellular membranes rather than with intracellular effects. He thought it possible, however, that the membrane changes could result in changes of the intracellular metabolism, possibly at the level of ribosomal RNA assembly. Two possibilities regarding lymphocyte stimulation by PHA were proposed; either the affinity

between the cell receptors and PHA varies from cell to cell, or different cells require saturation of different critical numbers of receptors before they are turned on. Receptor site studies showed PHA to bind irreversibly and very rapidly as opposed to Con A, which could be washed off the cells with a resulting loss of activity. He concluded that both mitogens were unlikely to act on the same receptors, but the same intracellular activities may occur in response to reactions between different cell receptor sites stimulated by the different agglutinins.

In one of the few reports published, concerning PHA stimulation of cell types other than leucocytes Smith and Goldman (1971) reported that both PHA and Con A induced multinucleated giant cells in human colostrum macrophage cultures, but not in mouse peritoneal macrophage cultures. Mouse peritoneal macrophages were found to have binding sites for PHA because of a macrophage interlinkage reaction which could be inhibited by the addition of N-acetyl-d-galactosamine. There were no binding sites found for Con A on the peritoneal cells. The authors found that the appearance of giant cells in cultures of human colostral macrophages upon addition of PHA or Con A revealed a striking distinction between these cells and mouse peritoneal macrophages. Human macrophages derived from 5-10 day old cultures of blood leucocytes responded to PHA in the same way as mouse peritoneal macrophages (no giant cell formation). However, after 10-25 days *in vitro* the human macrophages formed giant cells in response to PHA. This suggested that blood macrophages apparently differentiate in culture into cells with an enhanced potential for transformation into giant cells.

PHA Effect on Viral Replication

The effect of PHA on viral replication has only recently been investigated. Interest was first created in the PHA-viral relationship when Montgomery *et al.* (1967) demonstrated that the response of normal human lymphocytes to PHA, infected *in vitro* with rubella virus or Newcastle disease virus (NDV), was markedly inhibited (68-97% inhibition). They suggested that early association of lymphocytes with virus inhibits the function of the cell and that this phenomenon may be a means of detecting virus.

Rawls *et al.* (1967) used amantadine (Symmetrel), one of the first marketed antiviral drugs against certain lipid containing viruses (Hoffman *et al.*, 1965), to inhibit the mitogenic response of human lymphocytes stimulated with phytohemagglutinin. With both amantadine and rubella virus they found inhibition of the mitogenic response of the lymphocytes to PHA without a loss of the leucoagglutination effect. Because the mode of action of amantadine is the prevention of viral penetration, either by preventing pinocytosis or by altering the cell membrane (Hoffman *et al.*, 1965), Rawls *et al.* (1967) suggested that the drug inhibits PHA and viruses by a similar mechanism and that these data would lend further evidence to the theory that PHA acts primarily at the cell surface to stimulate lymphocyte mitogenesis.

Until 1963, replication of virus in human leucocyte cultures was limited to certain RNA viruses (Berg and Rosenthal, 1961, and Berg, 1961). Nahmias, Kilbrick, and Rosan (1964), however, found that the DNA virus Herpes simplex would replicate in human leucocyte cultures only in the presence of PHA. Since the virus would replicate only in

stimulated cells, the authors proposed that the leucocytes must be in a particular growth phase to be susceptible to the DNA virus.

A year later Duc-Nguyen and Henle (1966) observed the replication of mumps virus in human leucocyte cultures. They found that fresh leucocyte cultures, stimulated by mumps virus, NDV, and Senai virus, were capable of synthesizing interferon. The capacity to synthesize interferon in stimulated cells was lost after 24 hours incubation with all three viruses. In contrast to Wheelock (1965), they found no interferon-like inhibitor in PHA treated control lymphocytes. Wheelock (1965) had reported that a viral inhibitor, possibly degraded PHA, inhibited the cytopathic effects of Sendbis virus in human leucocyte cultures. The inhibitor was found to be similar to the interferon produced by NDV in leucocyte cultures except that it was labile to heat and to extremes of pH, whereas the NDV stimulated interferon was not.

Edelman and Wheelock (1968) found that when PHA was added to human, mixed leucocyte cultures and to lymphocyte cultures, small lymphocytes were converted into lymphoblasts and the susceptibility of lymphocytes to Vesicular stomatitis virus (VSV) was increased 30 - to 1,000 - fold higher than cells not treated with PHA. No increase was observed in monocytes or polymorphonuclear leucocyte cultures. Attempts were made to separate the lymphocyte viral enhancement properties from one of the three active components (leucoagglutination, inducer of RNA synthesis, and inducer of DNA replication) of PHA. Treatment of PHA by heat, trypsin, anti-PHA serum, ribonuclease, and deoxyribonuclease failed to separate the viral enhancement property, suggesting that it is not separate, but related to one of the above active properties. The

authors also observed that after PHA treatment of the lymphocytes an increased rate of RNA and protein synthesis occurred within 2 to 10 hours, whereas, an increased rate of DNA synthesis was not observed until after 24 hours. This indicated the possibility that VSV replication may be associated with the earlier change in RNA or protein metabolism.

The ability of vaccinia virus to replicate in human peripheral leucocyte cultures was shown by Miller and Enders (1968) when they found that vaccinia also required the presence of phytohemagglutinin in human leucocyte cultures for replication. Leucocytes not pretreated with PHA were found to form infective centers after infection but they did not host replication of infectious virus. They concluded that their data was in favor of the hypothesis that PHA induces intracellular changes in the mature lymphocytes which are essential for viral multiplication, rather than at the adsorption or penetration level of infection. Their results also indicated that the enhancement of viral growth was related to the blastogenic and mitogenic effects of PHA rather than to an unrecognized activity.

MATERIALS AND METHODS

Animals

Six to nine month old albino rabbits were employed for peritoneal macrophage harvests. They were purchased locally and observed at least one month prior to use in order to detect any unhealthy animals.

Culture Media

Primary chick embryo monolayers and rabbit peritoneal monocytes were cultured in Eagles Minimal Essential (MEM) media with glutamine. The culture media was supplemented with 2%, 5%, or 10% heated bovine sera, 10% sodium bicarbonate, and combiotics (200 units/ml of penicillin and 100 units/ml of streptomycin).

The sera used in the culture media was processed from bovine whole blood, donated and collected from the Fannestil Meat Packing Company, Emporia, Kansas. The blood was collected directly from the animal and allowed to clot at room temperature for 4 - 5 hours. The sera was then removed, centrifuged at 1500 rpm for 15 minutes and the supernatant prefiltered through four thicknesses of Whatman No. 1 filter paper. The filtrate was sterilized by filtration using Millipore positive pressure filter apparatus. The sterile serum was aliquoted into sterile serum bottles and heat inactivated for 30 minutes at 56 C. Before using the sera in rabbit peritoneal macrophage cultures, it was first streaked on Brain Heart Infusion Agar to test for sterility and tested for

compatibility in tissue culture by using it in growth media for mouse peritoneal macrophages and chick embryo fibroblast cultures. The serum was stored at -70 C.

Cell Cultures

a. Chick Embryo

Primary chick embryo tissue cultures were used for both viral propagation of stock virus and for quantitation of vaccinia virus.

Nine to eleven day old chicken embryos were harvested and trypsinized for one hour in 0.25% trypsin. The cell suspension was then filtered through sterile cheese cloth and the filtrate centrifuged at 2500 rpm for 7 minutes. If the chick monolayers were to be used for propagating stock virus, the cells were counted in an Improved Neubauer Haemacytometer and adjusted to 3×10^6 cells/ml in Eagles MEM with 5% serum. The cell suspension was then placed in Roux culture bottles, 50 ml/bottle, and allowed to monolayer at 37 C.

For monolayers used in titering the virus, the cell suspension was counted and adjusted to 2×10^6 cells/ml in growth media with 5% serum and placed in 1 oz. oval prescription bottles with 3 ml/bottles. For a maintenance medium after viral adsorption, Eagles MEM plus 2% serum was used.

b. Rabbit Peritoneal Macrophages

Rabbit peritoneal macrophages were obtained from rabbits injected 5 days prior to harvest with 50 ml of sterile mineral oil. The animals were sacrificed by injecting 5-10 ml of air intravenously into the marginal ear vein. The abdominal area was then saturated with a

disinfectant and shaved. The skin over the abdominal area was cut away and the exposed muscle swabbed with disinfectant.

For harvesting the peritoneal mononuclear cells, 100 ml of Hank's balanced salt solution (BSS), supplemented with combiotics and heparin (20 drops/100ml Hank's BSS), was injected into the peritoneal cavity. The abdominal area was then gently massaged and a small incision was made through the muscle wall and peritoneal lining to allow a 50 ml volumetric pipette to be inserted to collect the peritoneal fluid. The fluid was then transferred to a separatory funnel and placed at 4 C for 10 minutes.

After separation of the mineral oil and cell suspension, the cellular phase was collected and centrifuged at 1500 rpm for 5 minutes to pellet the monocytes. The supernatant was poured off and the cells washed with Hank's BSS. The cells were counted and resuspended in Hank's BSS to give a final concentration of 3×10^6 cells/ml. The cell suspension was distributed at 1 ml/tube in 13 X 100 mm culture tubes, each containing a 7 X 22 mm coverslip. The culture tubes were incubated at a 45° angle so the cell suspension would cover the entire coverslip. The cells were allowed to settle onto the coverslips for one hour at 37 C before the Hank's BSS was poured off and Eagles MEM with 10% serum was added.

Since there was no attempt to obtain pure cultures of a particular cell type in the peritoneal fluid, the peritoneal cells will be referred to collectively as macrophages, monocytes, or phagocytes.

Viral Propagation and Assay

a. Propagation

To provide vaccinia virus for the necessary experiments, the virus was propagated on chick embryo monolayers prepared in Roux culture bottles and concentrated by centrifugation. Just prior to complete monolayering of the chick fibroblasts, the growth media was poured off and the cells washed with Delbecco's phosphate buffered saline (PBS). The cells were then infected with vaccinia at a 0.09:1 virus-cell ratio, suspended in Delbecco's PBS + 0.1% bovine serum albumin (BSA). The virus was allowed to adsorb for 2 hours at 37 C with frequent, gentle rocking of the viral suspension for maximum adsorption. The virus inoculum was then poured off and replaced with maintenance media and incubated at 37 C for 48 hours or until development of a cytopathogenic effect (CPE). The bottles were then frozen and stored at -70 C.

To obtain cell-free virus, the monolayers were freeze-thawed three times to disrupt the cells and free the virus. The virus-cell suspensions were pooled and centrifuged at 3,000 rpm for 15 minutes to pellet the cell debri. The supernatant was taken off and frozen at -70 C, with an aliquote being saved for titering to determine the viral concentration.

Concentrations of between 5 X 10^7 plaque forming units/ml (PFU's/ml) and 1 X 10^9 PFU's/ml were maintained for stock virus. When the titer of the original supernatant containing the virus was assayed and determined to be below 5 X 10^7 PFU's/ml, the virus was concentrated by centrifugation in an International refrigerated vacuum centrifuge at 10,000 rpm for 60 minutes. The supernatant was poured off and the virus pellet

resuspended in an amount of Eagle's MEM + 10% serum that would provide a desirable concentration of virus. The suspension was then aliquoted and stored at -70 C.

b. Viral Assay

For determining the titer of virus, chick embryo monolayers were prepared in prescription bottles and washed with Delbecco's PBS before infection.

The viral systems to be titered were freeze-thawed three times to release cellular virus or freeze-thawed once if the system contained supernatant virus. Viral titer was determined by 10-fold serial dilutions of the viral suspension in Delbecco's PBS + 0.1% BSA. After a 2 hour viral adsorption at 37 C, the diluted virus suspension was replaced with maintenance media and the cultures were incubated for 48 hours to allow formation of plaques. The maintenance media was then poured off and the monolayers stained with a 1:10 dilution of Gram's crystal violet solution. Viral plaques were counted using a dissecting microscope and recorded as PFU's/ml.

All titering was done in duplicate and the average was used in recording the results. In titering the viral systems used in the rabbit macrophage cultures, two tubes and a duplicate of each tube were assayed, making the results the average of four separate samples.

Phytohemagglutinin

Phytohemagglutinin-P was obtained from Difco Laboratories as a sterile, dissicated material in 5 ml vials. Phytohemagglutinin in the dessicated state is stable indefinitely when stored below 10 C, but the rehydrated solution is stable at 2-10 C for only two weeks. The

smallest quantity available was in 5 ml amounts and because very small amounts of PHA were used in a single experiment, the stock solution was aliquoted in 1 ml amounts after rehydration and frozen for future use. Stock solutions in the reconstituted state were discarded after two weeks at refrigerator termperature.

Through personal communications with Difco Laboratories, it was learned that PHA is prepared and standardized according to its mitotic stimulatory activity rather than by weight. For this reason, the concentration of protein varied from batch to batch. Several batches of the commercial PHA were measured in.a Hitachi Spectrophotometer and the concentration of protein ranged from 12 mg/ml to 19 mg/ml.

Dilutions of PHA were made in Eagles MEM + 10% serum so they could be applied directly to the macrophage systems.

Concanavalin A

Concanavalin A (Con A) was obtained from the Nutritional Biochemicals Corporation in a 2X crystallized, saturated, sodium chloride solution that was carbohydrate free. The commercial solution contained 23.3 mg protein/ml. As with PHA, Con A was diluted in growth media to be applied directly to the cells.

Con A was stored at room temperature. It was found, however, that after about 10 days at room temperature, the optimum concentration used for our studies became toxic to the cell systems. Much lower concentrations were also found toxic to the cell cultures on storage of over two weeks. Because of the stability and inavailability of Con A, a limited amount of work was done with this agglutinin.

Staining Procedure

The stain used for microscopic observations in both chick embryo cultures and rabbit macrophage cultures was a modification of the May-Grunwald Giemsa Stain (Stumia, 1935). Coverslips were air dried in a vertical position and fixed in absolute methanol for 5 minutes. They were then stained in May-Grunwald for 9 minutes, followed by Giemsa for 14 minutes. The coverslips were then washed in acetone and transferred to acetone-xylene (50:50) for 1 minute and finally placed in xylene for 5 minutes. The coverslips were mounted in Permount solution on microscope slides for microscopic observations.

This staining procedure was also satisfactory in differentiating and counting mononuclear cells containing carbon in the procedure described below.

Test for Phagocytic Activity

To demonstrate the phagocytic properties of the peritoneal macrophage cultures, Higgin's India Ink was diluted 1:1,000 in Eagles MEM + 10% serum. This dilution assured a high phagocytic count of carbon particles in the mononuclear cells with a low concentration of background particles.

At the appropriate time periods, the growth media from the culture tubes was replaced with the diluted india ink solution. The tubes were incubated for 60 minutes at 37 C, in an inclined position, before the coverslips were removed and washed in Hank's BSS to remove the excess carbon particles. The coverslips were air dried and stained by the May-Grunwald Giemsa method.

Quantitation of the carbon phagocytized was done microscopically using the oil immersion lens. Phagocytic cells containing carbon were counted and recorded as the percent of total cells counted. Three different fields were counted for each coverslip and the average was used in recording results.

RESULTS

As previously stated, the purpose of this paper is to describe the effect of an inducer of DNA synthesis, phytohemagglutinin, on vaccinia virus, a DNA virus. Before attempting experiments involving viral infections, experiments were first performed to find the optimum concentration of PHA that would allow a maximum amount of agglutination of the host cells, rabbit peritoneal macrophages, without causing cell toxicity.

To determine the optimum concentration, a series of dilutions of the commercial stock PHA was made in growth medium and added to the macrophage cultures immediately after settling and left for 90 minutes. The media was then changed to untreated growth medium for 24 hours. Table I shows the results of microscopic observations of the concentration studies of PHA in rabbit peritoneal macrophage cultures. Maximum agglutination did not occur at concentrations lower than 25 ugm/ml and the PHA concentrations of 50 ugm/ml and higher showed a toxic effect in the cell system. Because of the maximum agglutination without showing a toxic effect to the macrophages, the concentration of PHA used in all the following experiments was 25 ugm/ml. Figures 1 and 2 illustrate the clumping effect of PHA at 25 ugm/ml and untreated control cells. The test cultures were treated with PHA for 90 minutes immediately after settling in Hank's BSS and then changed to untreated growth media for 24 hours.

Table I

The effect of PHA concentrations on
rabbit peritoneal monocytes after 24 hours

PHA concentrations (ugm/ml)	Amount of agglutination	Condition of cells
0	+[a]	normal
0.1	+	normal
1.0	+	normal
10	++	normal
25[b]	++++[c]	normal
50	++++	slightly injured
75	++++	injured
100	++++	injured
200	++++	injured

a. += least agglutination
b. optimum concentration
c. ++++= most agglutination

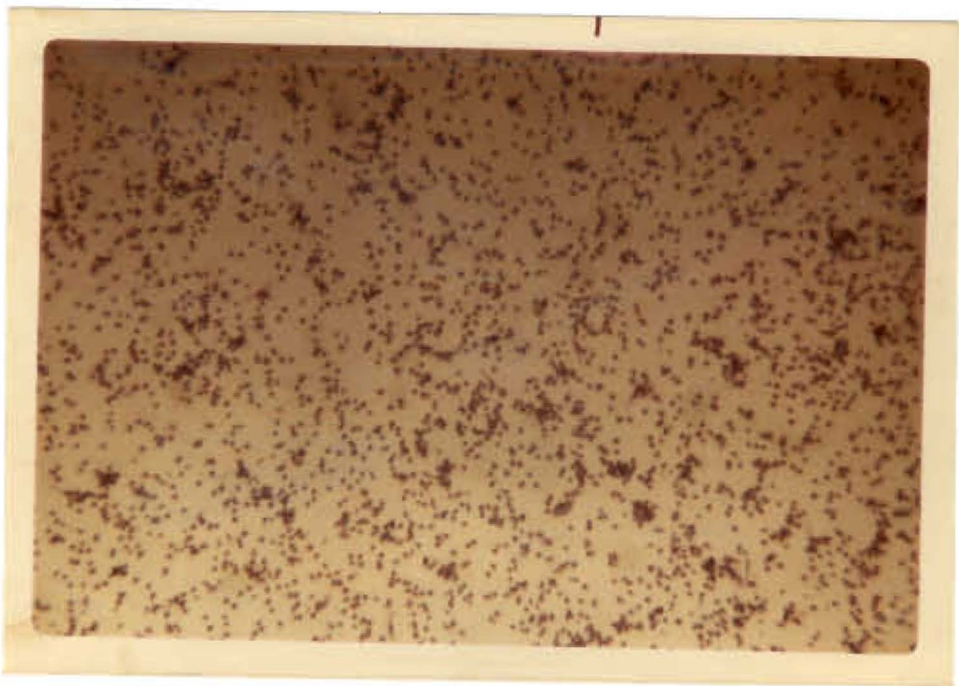

Figure 1. Normal rabbit peritoneal monocyte culture at 24 hours incubation. (100x)

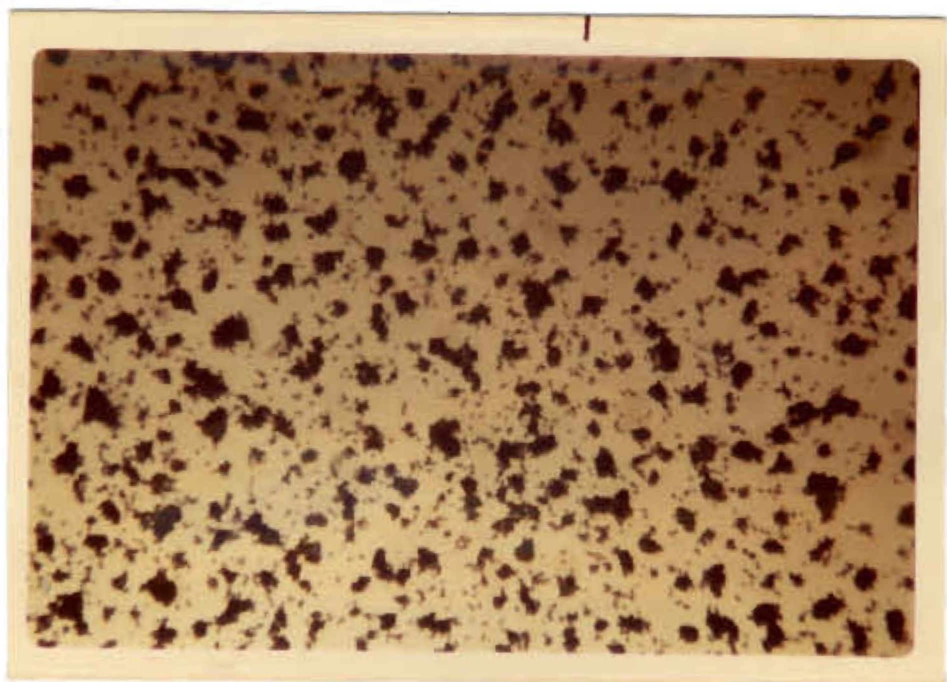

Figure 2. Clumping effect of PHA (25 ugm/ml) on rabbit peritoneal monocytes after 24 hours incubation. (100x)

Effect of PHA on Vaccinia Infection

After the optimal concentration of PHA was found, cultures of peritoneal macrophages were treated with PHA and infected with vaccinia to observe the effects of PHA on viral replication (Fig. 3). The experimental systems were designed to determine if there would be a difference in the effect of PHA on viral replication if the cells were treated with PHA for a short time prior to infection or if the cells were treated after viral adsorption, leaving the PHA in the media.

Figure 3 shows the results of PHA's effect on vaccinia replication in rabbit peritoneal macrophages. The control system consisted of culturing the cells in growth medium (Eagles MEM with 10% serum) for 90 minutes after a 1 hour settling period in Hanks BSS. The medium was then replaced with growth media containing virus at a 0.5:1 ratio of virus to cells. After a 2 hour adsorption period, the viral inoculum was replaced with untreated growth media. The two experimental systems were treated in a similar manner except one system contained PHA in the growth medium for 90 minutes prior to infection and the other system contained PHA in the medium which replaced the viral inoculum after adsorption. There was no attempt to make separate assays of the cellular and supernatant virus in this experiment. Thus, the titer values shown in Figure 3 reflect the titer of the virus in both the supernatant and cellular portions of the systems.

The results in Figure 3 show that at 24 hours post infection the viral concentrations were similar in all three systems. After 24 hours, however, there was evidence of viral replication in the untreated control cells but the replication seemed to be inhibited in both PHA

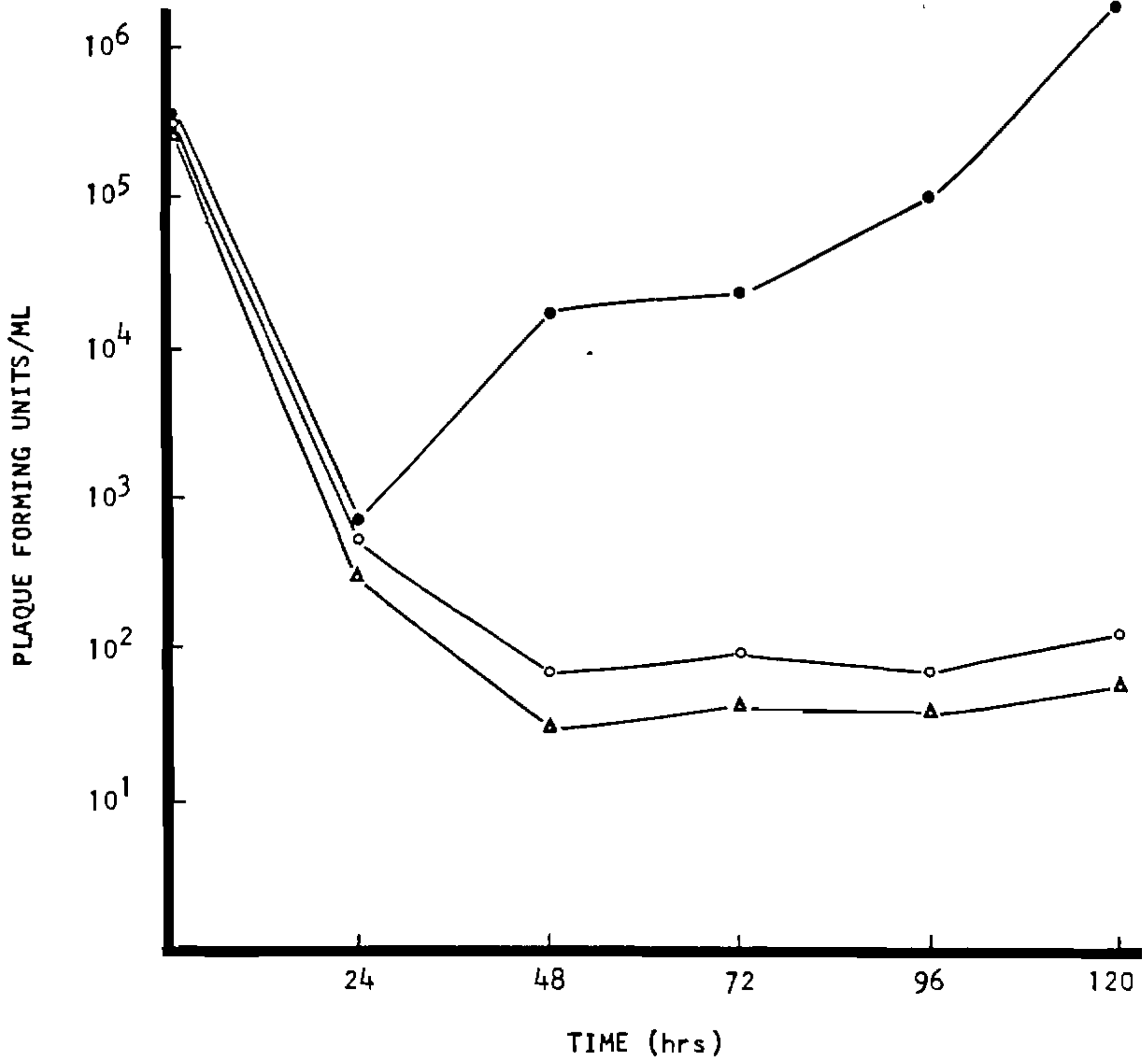

Figure 3. Effect of PHA (25 ugm/ml) on vaccinia replication in rabbit peritoneal monocytes. -•-, infected control; -o-, PHA added 90 minutes prior to viral adsorption; -▵-, PHA added after viral adsorption.

treated systems. The similarities in the inhibition of viral replication in both experimental PHA systems, suggested that there was little or no difference in inhibition whether the cells were treated with PHA prior to viral adsorption or treated immediately afterwards and remained for the rest of the experiment.

Two possibilities were regarded concerning the apparent inhibition of vaccinia replication by PHA. The first possibility was that PHA somehow inactivated the virus causing it to lose its infectivity. The other possibility was that PHA effected the host cell in such a way as to inhibit viral reproduction.

The first possibility was regarded as improbable after a survey of the literature indicated that PHA probably did not effect vaccinia by inactivating it (Miller and Enders, 1968). This supposition is also supported by later experiments cited in this paper. Since PHA probably does not effect the viral particle itself, it seemed apparent that the inhibition of vaccinia replication must be the result of PHA's action on the peritoneal macrophages.

To investigate this possibility, it was determined that a more detailed study of the mode of action of PHA in rabbit peritoneal macrophage cultures should be made.

Carbon Uptake

Because the cell membrane has been implicated in cell alterations caused by PHA, our first approach in studying the PHA macrophage interaction was to test the effect of PHA on the phagocytic activity of the monocytes. To simplify the complex system of the virus-cell interaction and so the phagocytic effects could be both observed and quantitated

microscopically, diluted india ink was substituted for the viral antigen.

The first attempt in measuring the phagocytic activity was designed to learn if the carbon particles would be phagocytized by the macrophage cultures in the presence of PHA. Of secondary importance was to find a relationship with the length of time of PHA treatment and the extent of inhibition of phagocytosis, if the theory of phagocytic inhibition were correct.

Two experimental systems were used for the above investigation. One system consisted of treating the cell cultures with PHA for 90 minutes, washing with Hank's BSS, and then replacing it with untreated growth medium. The other experimental system was designed to show the long term effect PHA would have on the macrophage cultures, thus, the PHA medium was not replaced but remained on the cells for the duration of the experiment.

The two experimental systems illustrated in Figure 4 clearly demonstrated the ability of PHA to inhibit phagocytosis of the carbon particles. The ability of PHA to inhibit the macrophage phagocytosis was shown to occur as early as 90 minutes after its addition. It was also shown that there was a marked difference in the length of time of inhibition between the two systems, suggesting that the longer the cell cultures were treated with PHA, the longer the period of maximum inhibition of phagocytosis.

A rather unexpected finding in the same experiment was the recovery of the macrophage cultures from phagocytic inhibition. This also seemed to be determined by the length of time the cells were treated with PHA.

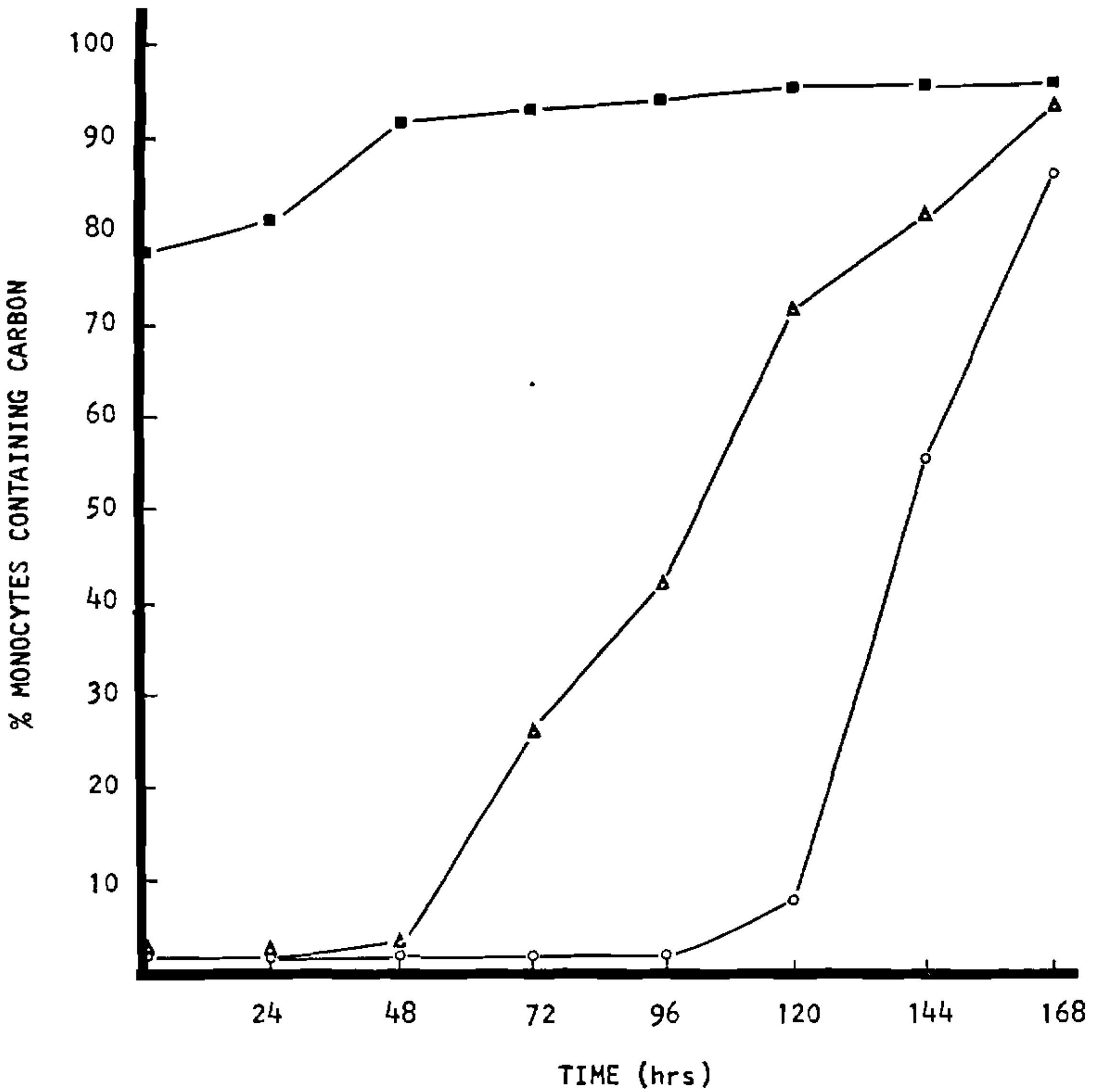

Figure 4. Effect of PHA on phagocytosis of carbon by rabbit macrophages. -■-, untreated control cells; -▲-, cells treated with PHA for 1.5 hours; -o-, cells treated with PHA for 168 hours.

As shown in Figure 4, the recovery period begins at 48 hours, when PHA is left on the cells for 90 minutes, and thereafter the cultures rapidly lose their inhibition and begin active phagocytosis of the carbon. However, the cultures with PHA left in the medium exhibited inhibition for about 96 hours before the cells began to recover, with the recovery process also occurring very rapidly.

Microscopic observations revealed that even after the experimental systems recovered from phagocytic inhibition and the number of phagocytic cells containing carbon was similar to that of the control cells, the quantity of carbon particles phagocytized in the test cultures was significantly lower in the PHA treated cells than in the control. Figures 5 and 6 show a comparison of control macrophages that are actively phagocytizing the carbon and PHA treated macrophages in which the phagocytic mechanism has been inhibited and are unable to phagocytize the carbon.

The results obtained from the above experiment confirmed that PHA did effect the macrophage cultures in such a way as to inhibit phagocytosis of carbon particles and that the length of time of inhibition and the time of recovery of the cells from the inhibitory effect was dependent on the length of time the cells were treated with PHA.

To provide a better understanding of the above results, the next several experiments were designed to obtain more detailed information on: (1) the relationship between the length of time the cells were treated with PHA and the time of recovery, (2) the nature of the recovery phase of the macrophages from PHA inhibition and (3) the action

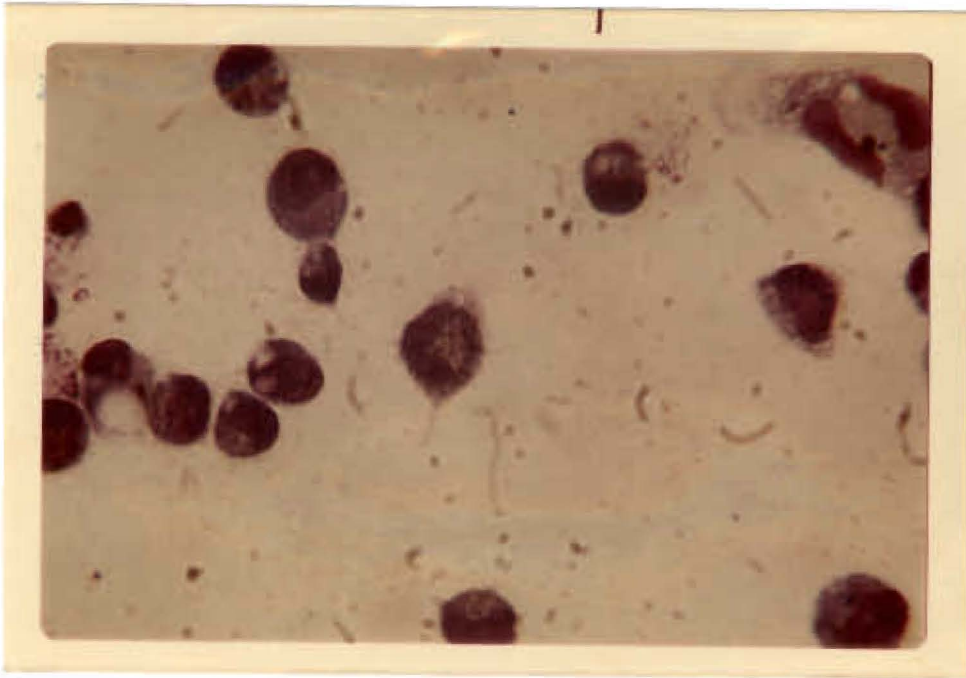

Figure 5. Untreated rabbit peritoneal monocytes showing phagocytosis of carbon particles at 24 hours. (970x)

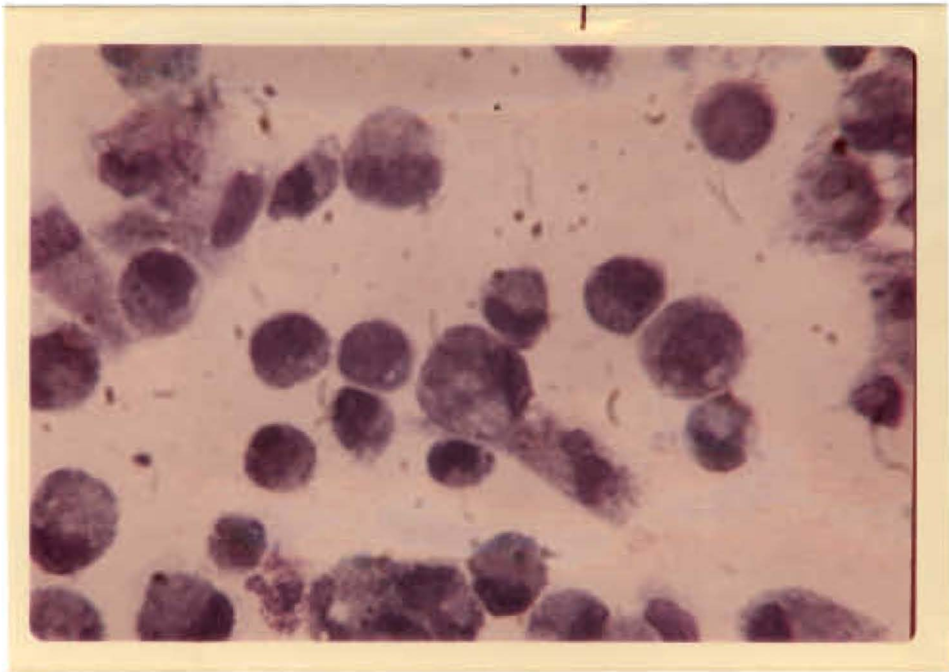

Figure 6. PHA treated rabbit peritoneal monocytes showing phagocytic inhibition of carbon particles at 24 hours. (970x)

of PHA on the cell surface membrane by comparing it with another agglutinin, concanavalin A.

Figure 7 represents a more detailed study on the time of treatment of the cells with PHA and the time of inhibiton before recovery. The results show the same effect as Figure 4, in that the longer the cells remained in contact with PHA the longer the inhibition of phagocytosis of carbon particles. Macrophage cultures were treated with PHA for 90 minutes, 24 hours and 168 hours. In another system the PHA treated media was poured off at 48 hours and fresh PHA treated media was applied and remained for the duration of the experiment. In the latter system the addition of fresh PHA caused a significant increase in the length of time of inhibition before recovery began to occur, as compared to the system where PHA was left in the media continuously for 168 hours.

These results confirmed that PHA does not have to be in the media continuously to produce the inhibitory effect and does not produce a permanent inhibitory effect in peritoneal macrophages. It also appears that because the recovery from inhibition can be post-poned by adding fresh PHA, there may be some type of breakdown or inactivation of PHA after a certain period of time.

A closely related problem was a study of the nature of the recovery period itself. In an attempt to account for the recovery period, three factors were investigated to find what effect they might have concerning the recovery of the cells from phagocytic inhibition. The factors examined were: (1) the presence or absence of the phagocytic inhibiting effect of PHA in the culture medium, during and after the

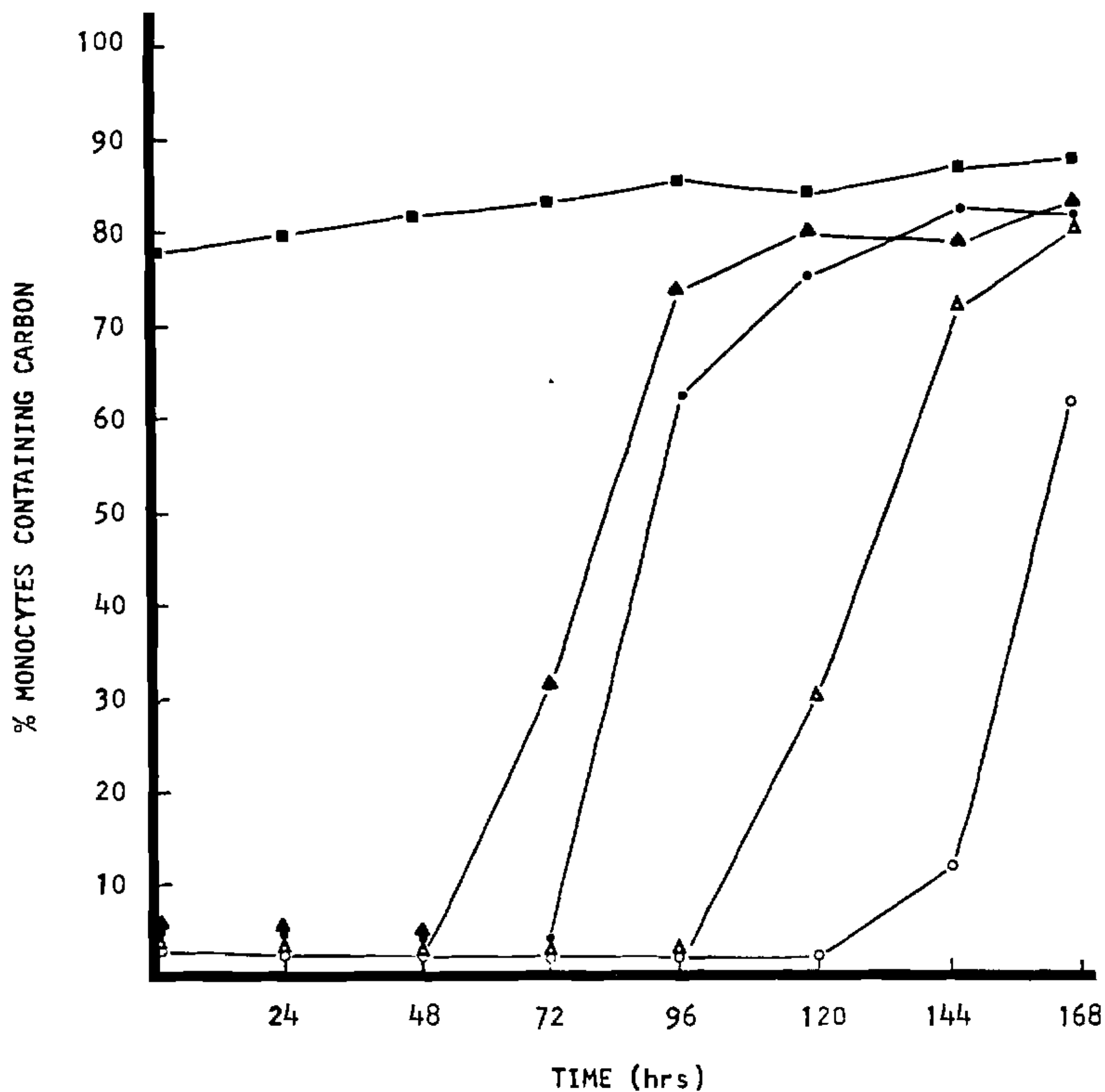

Figure 7. Comparison of time of phagocytic inhibition with time of PHA treatment. -▲-, PHA treatment for 1.5 hours; -•-, PHA treatment for 24 hours; -△-, PHA treatment for 168 hours; -○-, PHA treatment for 48 hours and changed to fresh PHA; -■-, untreated control.

recovery of PHA treated cells, (2) the stability of PHA's inhibiting effect upon prolonged incubation and (3) the effect of the age of the macrophage cultures.

Figures 8 and 9 illustrate the results of data collected from the above investigations. The graph in Figure 8 shows data collected from experiments designed to test the media for the presence or absence of the phagocytic inhibitory effect of PHA during the recovery period of the cells. To test the media for the effect of PHA, two control systems were used. One control was normal untreated macrophages while the other control consisted of cells in media treated and maintained with PHA to show the normal inhibition and recovery of the cells in culture. The experimental system chosen was a combination of untreated cells and PHA treated media from different incubation periods of the PHA treated cultures. Since the data desired was concerned with during and after the recovery phase, which did not usually occur before 72 to 96 hours in culture, the experimental system was not incorporated until the cultures were 48 hours old. In this way, results were obtained before, during, and after the cells recovered from phagocytic inhibition. After 48 hours incubation, PHA media from duplicate PHA treated macrophage cultures was transferred to duplicate 48 hour untreated cultures, from which the growth media was discarded just prior to the transfer. These cultures, with untreated cells and PHA treated media, were then incubated for an additional 24 hours before being exposed to carbon and assayed for phagocytic activity. The data from this example are shown at 72 hours in Figure 8. It should be noted that the data presented for the experimental system actually represents the PHA activity of the

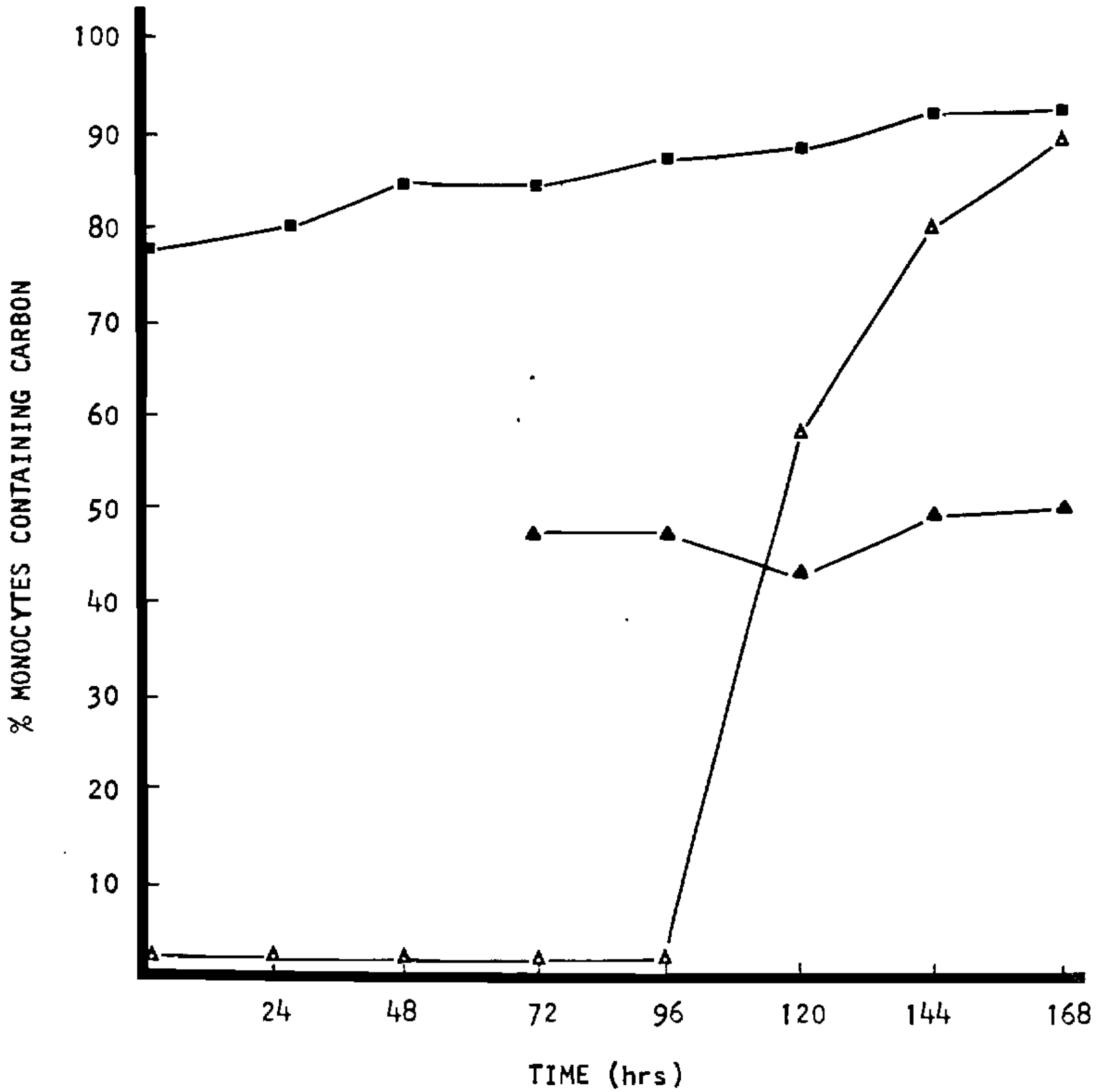

Figure 8. Relationship of phagocytic activity to unadsorbed PHA. -■-, untreated control; -△-, PHA control; -▲-, media from PHA treated macrophage cultures, transferred to untreated cells for 24 hours.

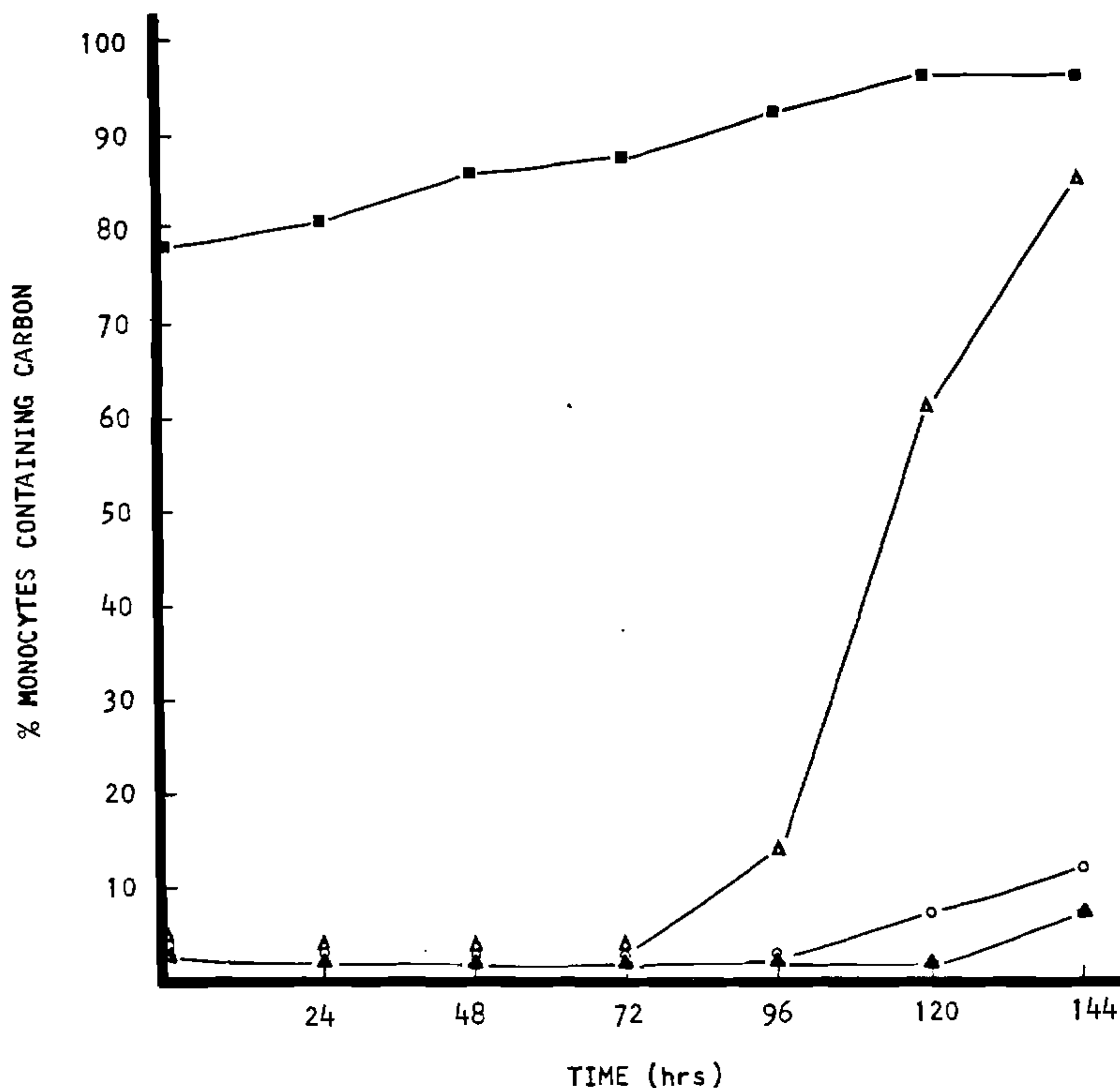

Figure 9. Effect of phagocytic activity of macrophage cultures to prolonged incubation of PHA, and the effect of their age to freshly prepared PHA media. -■-, untreated control; -△-, PHA control; -o-, PHA media incubated without cells, then applied to untreated cells for 1.5 hours; -▲-, freshly prepared PHA media applied to untreated cells for 1.5 hours.

unadsorbed PHA from cultures 24 hours prior to the time they were assayed.

Figure 8 shows a significant difference in the percentage of phagocytosis between the experimental system and both controls indicating that the media still contained PHA capable of inhibiting phagocytosis during the recovery and post recovery of the cells from inhibition. The rather constant inhibition level at the times tested for the experimental system suggested that there was a constant amount of PHA taken up by the cells with no significant change in PHA activity during the recovery phase.

In the next experiment, prolonged incubation of PHA and the age of the macrophage cultures were investigated to determine if either had an effect on the ability of the cells to recover from phagocytic inhibition (Fig. 9). Untreated macrophage cultures served as one control and PHA treated cultures served as the other control in the experiment. PHA treated media with no cells served as the experimental system for the investigation of the effect of prolonged incubation. The treated media was treated identically to the other systems containing cells and all were incubated at 37 C. Every 24 hours a sample of the treated media without cells was transferred to untreated cells incubated the same length of time. The cells and treated media were incubated an additional 90 minutes to allow the PHA sufficient time to adsorb to the cells, after which they were treated with carbon and the percentage of phagocytosis calculated. The data shown in Figure 9 illustrates that upon prolonged incubation of the treated media, the PHA was still fully capable of inhibiting phagocytosis of the cells during and after the

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recovery period of the PHA control system and only at 120 hours was there evidence that the inhibitory effect was being diminished.

Also shown in Figure 9 are results showing the effect of the age of the macrophage cultures to PHA treatment. These were determined by adding freshly prepared PHA treated media to different untreated cultures at 24 hour intervals for 144 hours. The fresh PHA was allowed to adsorb for 90 minutes before the cultures were tested for phagocytic efficiency. The results were very similar to the other experimental system (effect of incubation) except that the phagocytic inhibition was kept at a maximum about 24 hours longer before an indication of recovery was observed.

From the results obtained in Figure 9, both inactivation of PHA by prolonged incubation and the age of macrophage cultures were eliminated as possible causes for the macrophage recovery from the inhibitory effect. Even though the age of the macrophage cultures was not thought to be a determinent in the recovery process, it was found to have some effect on the agglutination of cells treated with PHA. Microscopic observations revealed that older macrophage cultures tended to clump or agglutinate in smaller and much tighter clumps than the younger cultures when fresh PHA was applied for short lengths of time.

The last type of study with the PHA-macrophage system and carbon particles, was an attempt to determine if the ability to produce phagocytic inhibition was common to another plant agglutinin or if it were specific for PHA. Concanavalin A was used as the comparative plant agglutinin. Using the same procedure for determining the optimum concentration of PHA for rabbit peritoneal monocytes, Con A was found to

provide the best agglutination of the peritoneal cells, without toxicity, at 103 ugm/ml.

The first experiment in the comparison studies was to determine what effect Con A had on phagocytosis of the peritoneal macrophages (Fig. 10). Two control systems were used, one containing untreated cells and the other containing PHA treated cells. The three experimental systems were: (1) cells treated with Con A for 90 minutes, (2) cells treated with Con A for 24 hours, and (3) cells treated with Con A for 168 hours. In the first two experimental systems, the media was changed to untreated growth media after appropriate periods of Con A treatment.

The results of the above experiment, shown in Figure 10, indicate there may have been some phagocytic inhibition due to the presence of Con A in the media, but not to the extent observed in the PHA systems. There were no significant differences in phagocytic activity between the different Con A experimental systems as there were with PHA and no abrupt recovery period was seen in the Con A treated cells.

Since Con A was shown to have very little inhibitory effect on phagocytosis, it seemed probable that the two agglutinins acted on different cell receptor sites. If this were true, there should be no competition between the two in producing phagocytic inhibition in macrophage cultures. The inhibition, characteristic of PHA, should be distinguishable whether PHA and Con A are added simultaneously or added separately at different times in the same cell cultures.

To test this, PHA and Con A were added simultaneously after the macrophage cultures had settled and the phagocytic activity was measured

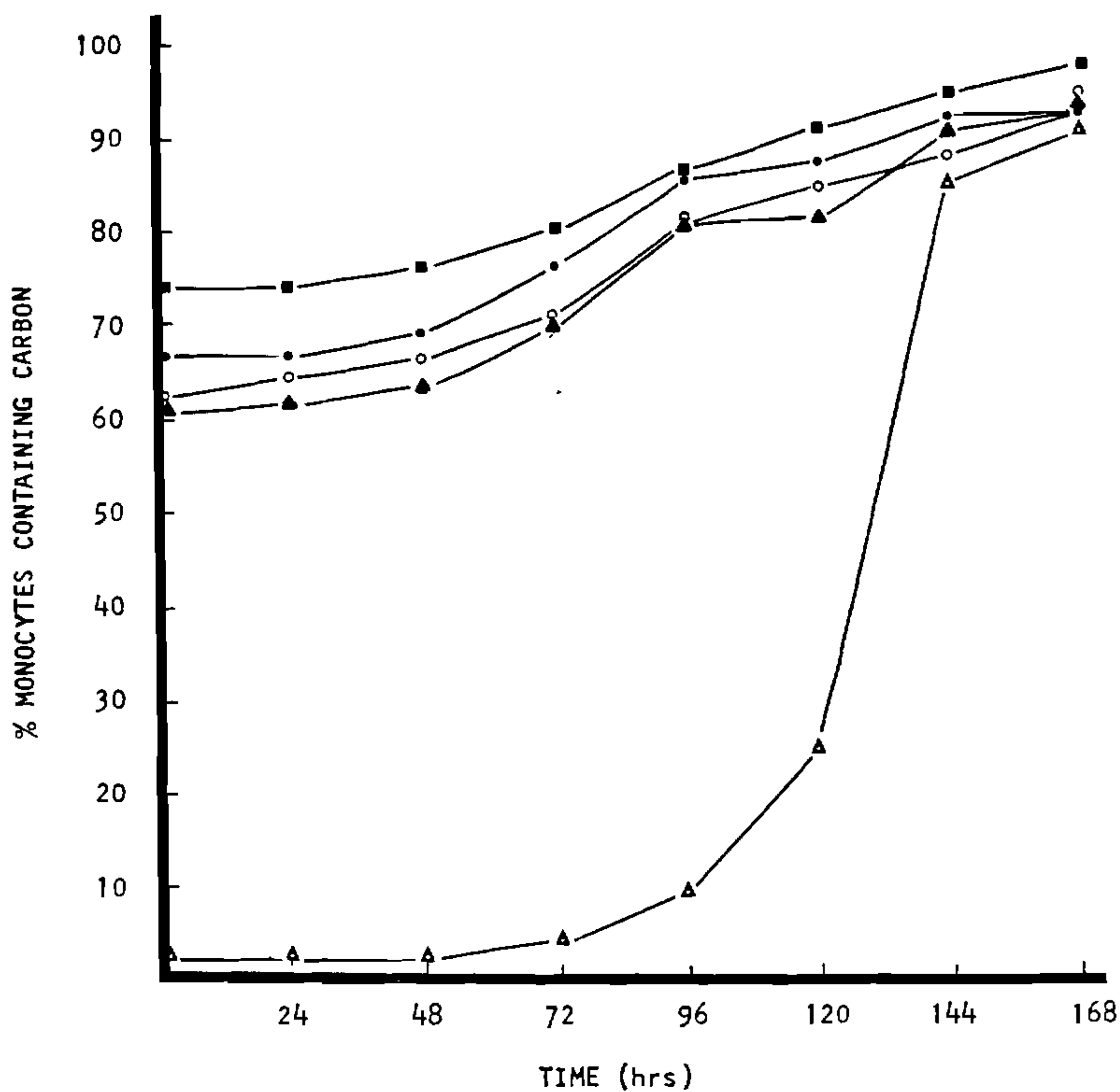

Figure 10. Comparison of phagocytic activity of Con A (103 ugm/ml) and PHA (25 ugm/ml) on rabbit peritoneal mononuclear cells. -■-, untreated control; -•-, cells treated with Con A for 1.5 hours; -o-, cells treated with Con A for 24 hours; -▲-, cells treated with Con A for 168 hours; -Δ-, PHA control.

at 90 minutes and at 24 hour intervals therafter (Fig. 11). Table II shows a summary of some of the results of a more detailed investigation of the competition between the two agglutinins with the addition, at different times, of PHA and Con A to the same cell cultures.

The results of both Figure 11 and Table II show that Con A does not interfere with the ability of PHA to effectively inhibit phagocytosis. The PHA-Con A system illustrated in Figure 11 showed a shorter inhibitory response than the PHA control, but in general, the data of the test system was very similar to the PHA response, as indicated by the PHA control.

The different systems shown in Table II were designed to determine the effect of phagocytic activity when media treated with PHA was added to a culture and then changed to media treated with Con A. Systems were also used involving the reverse applications of the two agglutinins, with Con A being added first and then changed to PHA. By this type of an experiment it was thought that we could determine if one agglutinin would mask the effect of the other, with particular concern to Con A masking the ability of PHA to induce phagocytic inhibition. The times of application of each agglutinin in the cultures was determined so a comparison might be made with other PHA experimental systems when time of application and the duration of PHA treatment was a factor (Fig. 4, 7). The results showed a definite inhibition of phagocytosis, characteristic of PHA's effect on the cells, in all systems containing PHA. This was indicated by the very low percentage of carbon uptake by the treated cultures. The 36% carbon uptake shown in the system containing PHA for 96 hours and Con A for 24 hours is indicative of the

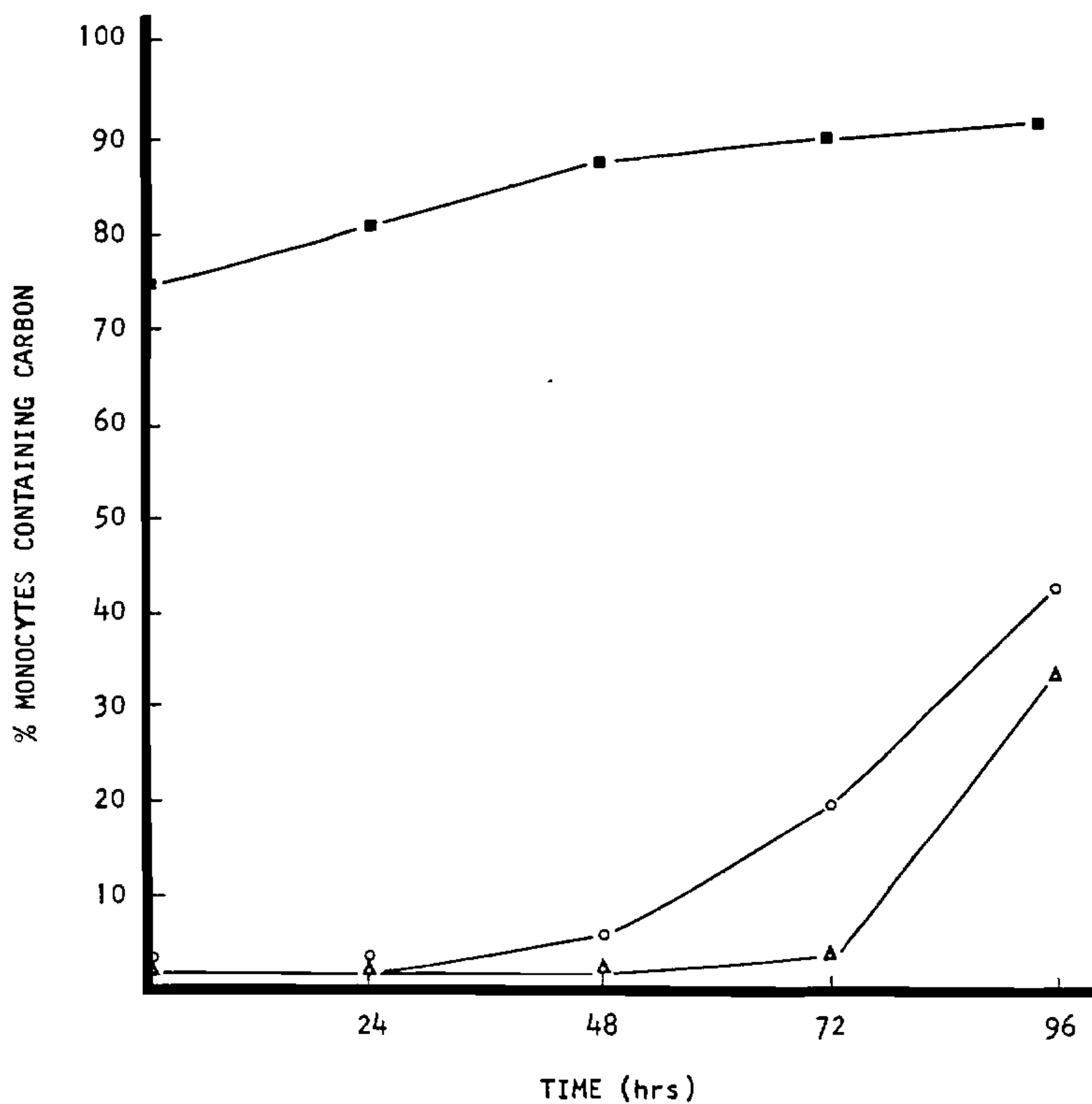

Figure 11. Effect on phagocytic activity of peritoneal macrophages with PHA and Con A added simultaneously. -■-, untreated control; -▲-, PHA control; -○-, PHA and Con A added simultaneously.

Table II

A. Percent carbon uptake in rabbit peritoneal monocytes treated with both PHA and Con A

Combinations used	Total hours incubated	Percent carbon uptake
PHA (90 min) —[a] Con A (90 min)	3	1[b]
PHA (24 hrs) — Con A (90 min)	25.5	1[b]
PHA (48 hrs) — Con A (24 hrs)	72	3
PHA (72 hrs) — Con A (24 hrs)	96	7
PHA (96 hrs) — Con A (24 hrs)	120	36
Con A (24 hrs) — PHA (24 hrs)	48	1
Con A (24 hrs) — PHA (72 hrs)	96	5
Con A (48 hrs) — PHA (90 min)	49.5	1[b]
Con A (72 hrs) — PHA (90 min)	73.5	2

a. changed to
b. less than 1%

B. Percent carbon uptake in control monocytes

Controls	Time (hrs) 1.5	24	48	72	96	120
Untreated	75%	83	86	90	92	90
PHA	1[a]	1[a]	1	1	5	41
Con A	62	65	74	81	88	90

a. less than 1%

middle of the recovery period. This is also indicated by data from the PHA control

Direct Effect of PHA on Vaccinia

Before investigating the effect PHA had on vaccinia replication in the macrophage cultures, it was first necessary to find if PHA had any direct effect on the virus which might effect the outcome of later results. To do this, virus was added to PHA treated media in the absence of cells and incubated at 37 C. At selected time intervals the media was frozen until the experiment was completed and then all the samples were titered to give the results shown in figure 12. The time periods chosen for titering were selected so the results might be more easily compared to the results of the following experiment (Fig. 13 and 14). Figure 12 also shows the control, consisting of untreated media plus virus, and the comparative agglutinin system, Con A plus virus. Both the latter systems were also incubated without cells.

The titers from both the untreated and PHA treated media showed a slight decrease from 0 hour to 24 hours which was expected since there was no viral host. However, there was no significant difference in the change of titers of the two systems and their similarities suggested that PHA does not inactivate the virus.

The Con A system seemed to maintain viable virus for a longer period of time than the other systems, but there were no determinations as to its significance.

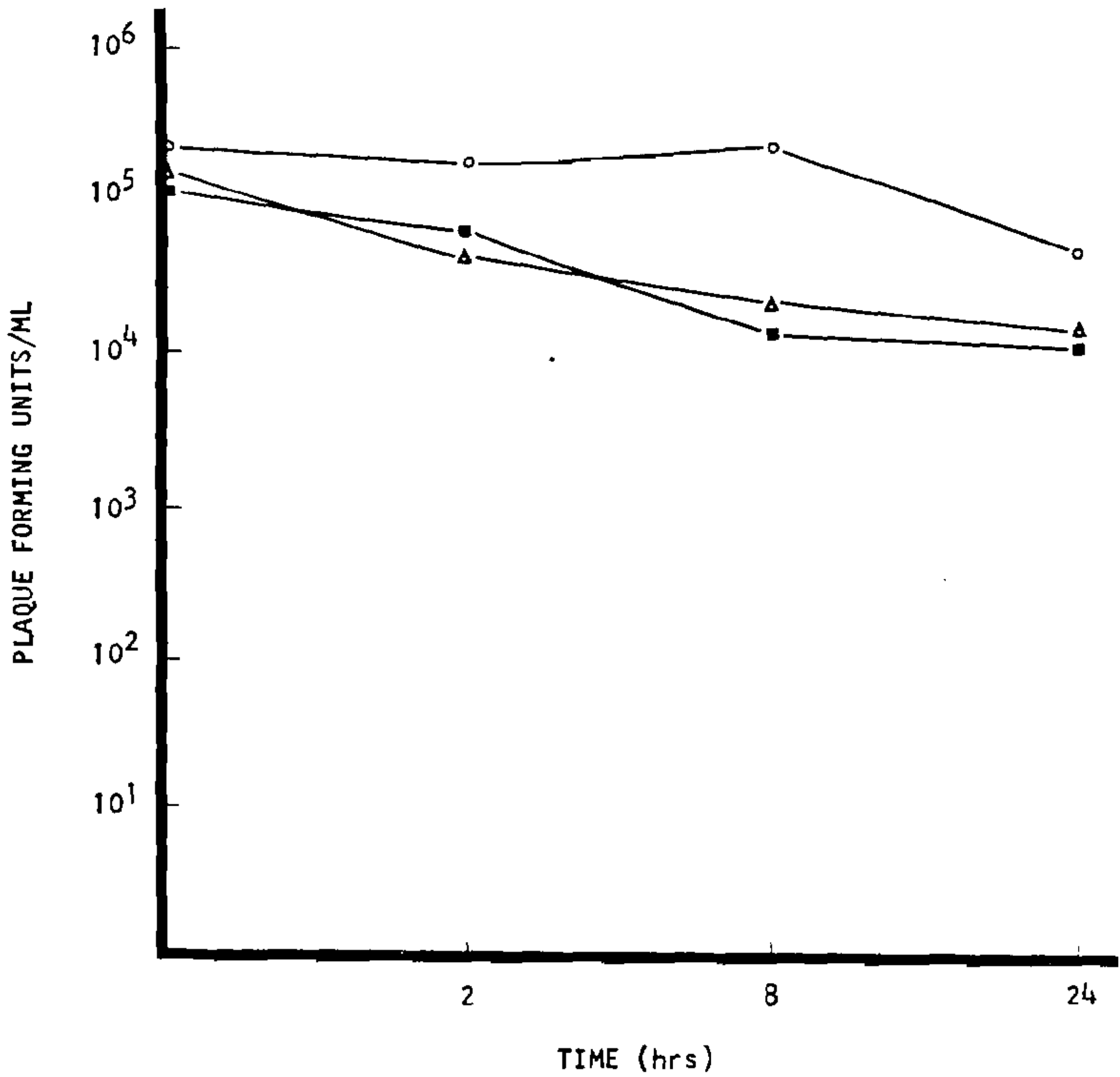

Figure 12. Effect of PHA and Con A on vaccinia virus. -■-, virus in untreated growth media; -▲-, virus in PHA treated media; -○-, virus in Con A treated media.

Viral Inhibition by PHA

To find if the virus was being phagocytized in PHA treated systems or if it were being inhibited from phagocytosis and left free in the media, experiments were conducted to assay for the presence of virus in both the supernatant and the cellular portion of the cultures.

Because the first experiment showing viral inhibition in PHA treated cells was a long term experiment (Fig. 3) with the first viral assay not being made until 24 hours, it was decided that in testing for viral phagocytosis, assays should be made before 24 hours so the fate of the initial viral inocula in the PHA treated cultures could be determined and compared with infected untreated cultures.

To determine this, the next experiment involved making viral assays of both the supernatant and cellular portion of the control, PHA and Con A systems. The assays were made at the following times: (1) immediately after adsorption of the virus to the cells (2 hours), (2) at an intermediate period of the viral cycle, before mature viral particles were formed (8 hours), (3) at 24 hours, giving sufficient time for mature particles to have been released. Con A was again used as a comparative system because of its phytogenic properties and its inability to produce phagocytic inhibition.

Figure 13 shows the results of the viral assay in the supernatants of all four systems. As expected, the viral titer from the supernatant of the infected control cells diminished at 2 hours and at 8 hours indicating the subsequential uptake and processing of the virus by the phagocytic cells. The titer values began to level off at 24 hours. showing the completion of the viral life cycle and reinfection. As was

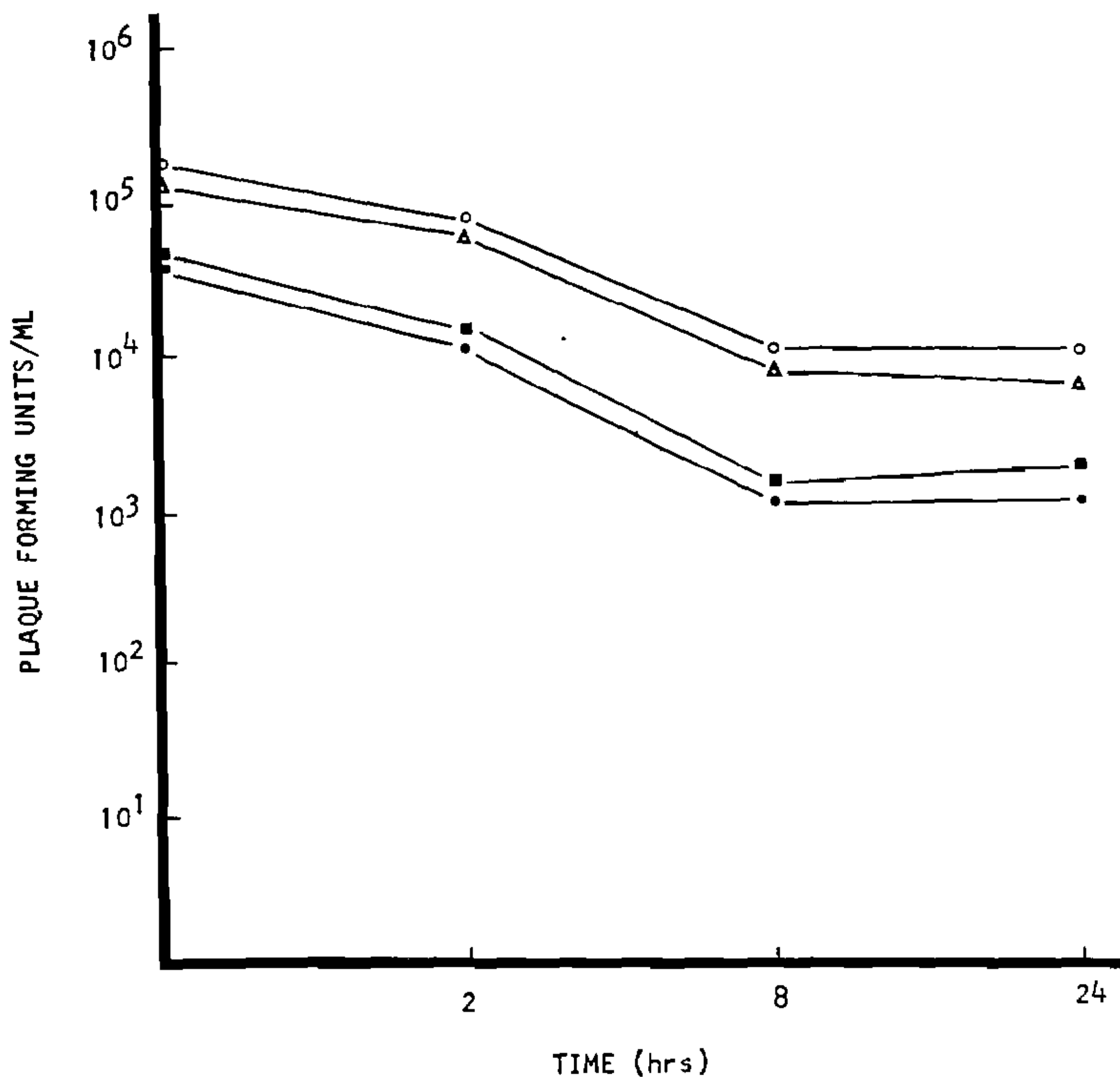

Figure 13. Viral assay from supernatant of infected, PHA treated peritoneal macrophage cultures. -■-, untreated control; -•-, cells treated with PHA 90 minutes before infection; -▲-, cells treated with PHA 24 hours before infection; -o-, cells treated with Con A 90 minutes before infection.

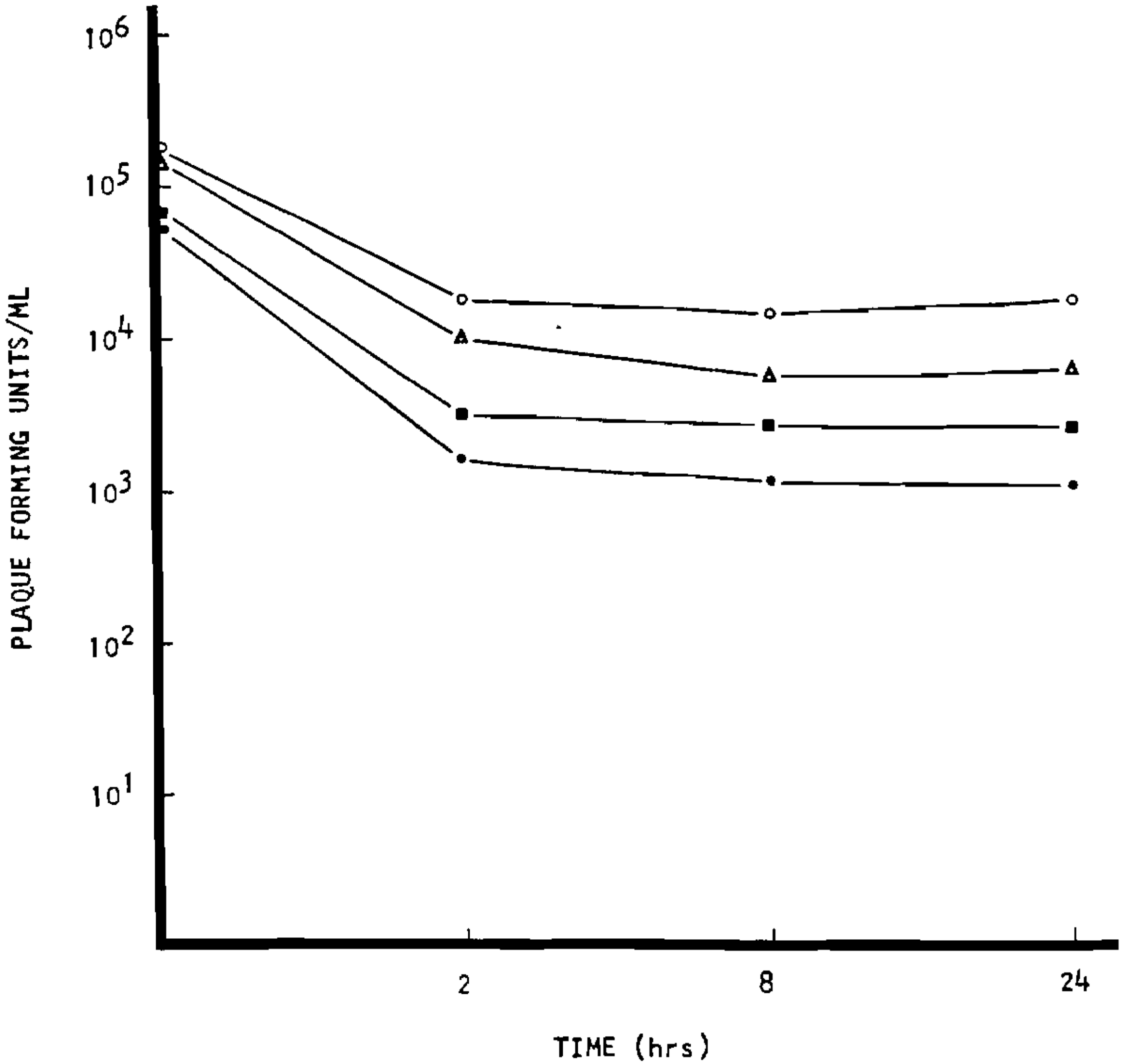

Figure 14. Viral assay from cellular portion of infected peritoneal macrophage cultures. -■-, untreated control; -•-, cells treated with PHA for 90 minutes before infection; -o-, cells treated with PHA for 24 hours befor infection; -▲-, cells treated with Con A for 90 minutes before infection.

also expected from the observations on carbon uptake, the viral titers from the Con A system were similar to that of the control. However, the similarities between the viral titers of the PHA treated systems and the control system were not expected. This indicated that the virus was being phagocytized in a manner similar to that of the virus in the untreated and Con A treated cells.

The cellular virus assay shown in Figure 14 supports the evidence shown in Figure 13, indicating that the virus is not being inhibited from phagocytosis by the macrophages but that it does gain entrance into the cells. This is indicated in Figure 14 by the parallelism of the titers of the PHA treated systems with that of the control and Con A systems. Like the viral titers in the supernatant, viral assays of both PHA systems in the cellular portion indicated a normal infection and replication up to 24 hours.

The data collected from Figures 13 and 14 suggested that the viral inhibition in peritoneal macrophages treated with PHA was not due to an inhibition of phagocytosis of the virus as was suggested by the experiments utilizing carbon. They also indicated that there was no significant difference in the viral titers between the system infected after a 90 minute treatment with PHA and the system which was infected after PHA treatment for 24 hours, suggesting that there were no viral inhibitors produced by the PHA treated peritoneal macrophage cultures.

DISCUSSION

Recent investigations of viral replication in PHA treated lymphocytes have been chiefly concerned with the ability of PHA to stimulate reproduction in cells previously unable to host the virus. Our results, however, indicated that PHA treated rabbit peritoneal phagocytes did not stimulate vaccinia virus replication, but inhibited it. Since these results contradicted much of the work done with viral infected PHA treated lymphocytes, our study was designed to obtain a better understanding of the effect that PHA has on the vaccinia-macrophage relationship.

Investigations of PHA in peritoneal macrophage cultures indicated a much smaller concentration of PHA was required to agglutinate the macrophages than was normally used for lymphocyte studies. Whereas, our optimum concentration was found to be 25 ugm/ml, most of the work with PHA treated lymphocytes was either around 200 ugm/ml (Miller and Enders, 1968) or, as in most of the studies, the concentration was indicated as a certain portion of commercially prepared PHA. Since the commercially prepared PHA is standardized for mitogenic activity, rather than protein content, an effective comparison with lymphocyte studies cannot be made.

The initial experiment, resulting in inhibition of viral replication in infected PHA treated macrophages, showed that there was no difference in the viral inhibition if the macrophages were treated with PHA

for a short time prior to infection or if the cells were treated for a long period of time after viral infection. This indicated that PHA did not have to remain in the media to prolong the inhibition and that the cells did not have to be pretreated with PHA to stop viral reproduction. It is interesting that Miller and Enders (1968) found that both pretreatment of the cells and the continuous presence of PHA was required for vaccinia replication in peripheral blood leucocytes.

Because of PHA's implications in cell membrane changes causing the agglutination of red blood cells (Rigas and Osgood, 1955), leucocytes (Hirschhorn et al., 1963), and tumor cells (Tunnis, 1964) the inhibition of viral replication was thought to be a result of a membrane change on the PHA treated macrophage. Since no viral particles could be detected, leaving the host cell, it seemed probable that the virus was unable to gain entrance into the cell and initiate replication. It seemed logical to initiate a study of viral inhibition by first examining the phagocytic mechanism in cells treated with PHA. Carbon particles from diluted India ink were substituted for the virus as a model for the virus-macrophage system in the phagocytic studies. This was done for reasons of simplicity, since it allowed the investigator to observe the ingestion of the colloidal material with a light microscope and also allowed rapid quantitative measure for the results.

Since it was not known what effect the length of PHA treatment would have on the macrophages, both a short term (90 min) exposure and a continuous exposure (168 hrs) of PHA were investigated. The results showed that during both exposure times there was an almost total inhibition of phagocytosis of carbon particles by the PHA treated cells.

An unexpected finding, however, was the ability of the treated cells to recover from phagocytic inhibition. The recovery time was found to be dependent upon the length of time of PHA treatment. The cells which had been exposed to PHA for only a short time recovered much faster than the cells which had been continuously exposed to PHA. Recovery from the phagocytic inhibition was usually very rapid and there seemed to be an almost complete recovery of phagocytic activity. This suggested that the effect of PHA on the macrophages was reversible and that it apparently did not induce permanent damage to the phagocytic mechanism. The only evidence to the contrary was microscopic observations which indicated that cells that have recovered from PHA treatment seemed to phagocytize a smaller amount of the carbon than the untreated cells.

The time of PHA treatment of the cells was shown to be a determining factor for the length of phagocytic inhibition and the time at which recovery from inhibition occurred. Because the cells were eventually capable of recovering from inhibition, even when the PHA was left in the media continuously, it seems probable that PHA may lose its ability to inhibit phagocytosis. This is supported by evidence that fresh PHA media, substituted for PHA media in culture for 48 hours, can prolong the phagocytic inhibition of the monocytes. Perhaps the loss of PHA's inhibitory effect was due to prolonged incubation of PHA with a resulting inactivation of the PHA molecules, aging of the cells in culture, the cells own defense system, or possibly a combination of these factors.

It seemed logical to assume that elucidation of the mechanism of recovery of macrophages from PHA treatment might help explain the mode

of action in the inhibition of vaccinia replication in the peritoneal mononuclear cells.

Several theories were investigated in an attempt to find an explanation for the recovery from phagocytic inhibition in PHA treated phagocytes. Our data indicated that neither the prolonged incubation of PHA nor the age of the macrophage cultures were independently responsible for the recovery process. Although both systems eventually showed the beginning of an apparent recovery from phagocytic inhibition, it was a gradual response and occurred later than the more abrupt recovery response under investigation. It should be noted, however, that in both systems the results were obtained by a pulsing procedure, where PHA treated media, either incubated or freshly prepared, was incorporated for only 90 minutes at each time interval tested before the cultures were assayed for phagocytic activity. This means that the effect of the long term PHA-macrophage association was not taken into consideration. It is possible that when the cells have been treated for periods exceeding 90 minutes that the prolonged incubation of PHA or the age of the macrophages may act in an indirect way or in conjunction with the length of PHA treatment in facilitating the recovery process.

The possibility of the PHA molecules disassociating after a certain length of time, leaving the cell undamaged and capable of recovery from phagocytic inhibition, seemed unlikely since Barberg et al. (1966) showed that PHA possessed an irreversible binding to leucocytes and could only be disassociated by the addition of N-acetyl-d-galactosamine. They also showed that after the disassociation, neither the cell nor the PHA molecules were damaged. Lindahl-Kiessling and Mattsson (1971) also

demonstrated the irreversible binding effect of PHA to lymphocytes in studying the effect of metabolic inhibitors in agglutination of lymphocytes.

The possibility of the media becoming depleted of PHA, thereby causing the recovery from inhibition, was thought to be unlikely since the PHA could be detected before, during, and after the macrophages had recovered from the inhibition. If it were a matter of PHA disassociating from the cell during recovery then it would have been expected to find an increase of unbound PHA with an increase of phagocytic inhibition during the recovery period. This was not found, however, lending support to perhaps a more complex mechanism.

The fact that the unadsorbed PHA (Fig. 8) did not inhibit phagocytosis to the same extent as the PHA control was probably due to most of the PHA in the media becoming cell-associated, thereby diluting the concentration of the unbound PHA molecules left in the media to be transferred to the untreated cells. The lack of a significant change in phagocytic activity during the different times that the unbound PHA was tested suggested that there may be a constant amount of PHA that becomes cell associated, leaving the remaining unbound PHA in the medium still capable of inhibiting phagocytosis.

Since the concentration of the unbound PHA apparently does not change, then it is likely that there is either no disassociation of the PHA from the macrophages, or if the PHA does disassociate, the PHA molecules are rendered incapable of producing a phagocytic inhibitory effect on other macrophages. The latter reasoning, however, is in contradiction with Barberg et al. (1966) and their work with PHA-leucocyte systems.

The PHA which was initially adsorbed may remain on the cells, occupying all the PHA receptor sites, even though the cell has recovered from the inhibition. It is also possible that the PHA molecules could alter the receptor sites enough that the cell would not "recognize" additional PHA and then be slowly taken up by the cells, thus allowing them to recover from phagocytic inhibition. The ability of lymphocytes to gradually take up the PHA molecules over a prolonged period (72 hrs) was suggested by Lindahl-Kiessling and Mattsson (1971). They also demonstrated that the uptake of PHA was not actively transported by an energy requiring mechanism such as endocytosis.

Fluorescent labelling of PHA would seem to be a useful tool in following PHA adsorption to the macrophage and to clarify the possibility of PHA remaining on the cell surface during their recovery from phagocytic inhibition. Several investigators (Michalowski et al., 1964; Michalowski et al., 1965; Razavi, 1966) used this method for following PHA treatment of lymphocyte cultures. In these investigations, however, PHA was shown to localize in different areas of the cell. The type of lymphocyte affected was even controversial. Berman and Andrews (1970) suggested several possible reasons as to the contradictions of results in PHA localization. One such possibility was that conjugation with FITC may change either the whole PHA macromolecule or only a part of it. Another possibility was that only a part of the PHA macromolecule may actually become labelled. Since there are at least two separate, distinct parts of the molecule, agglutinin and mitogenic (Nowell, 1960), it is feasable that only the portion that enters the cell is labelled, leaving the agglutinating portion unlabelled and on the cell surface.

Because of the uncertainty of the mechanism of FITC conjugation with PHA this method of observation was not used in our results.

Comparison studies using concanavalin A suggested that the inhibitory effect PHA induced in the phagocytosis of colloidal carbon is not a phenomena manifesting all plant agglutinins. Con A was used as the comparative agglutinin because of several of its properties that are similar to those of PHA. It is a plant lectin that agglutinates erythrocytes (Sumner and Howell, 1936), induces lymphocyte transformation to blast cells (Novogradsky and Katchalski, 1971), and has been shown to alter the cell membrane after adsorption to lymphocytes (Inbar _et al_., 1972). Unlike PHA, however, Con A is especially important because of its specificity in agglutinating malignant cells (Inbar and Sachs, 1969). Con A was also used because of the implication that it either adsorbs to different lymphocyte cell receptor sites than PHA or it has a different type of binding to cells (Lindhal-Kiessling, 1972).

The phagocytic studies of Con A treated macrophages were made in a similar manner as were made for PHA. Our results showed that concanavalin A did not inhibit macrophage phagocytosis of carbon particles to the same extent as did PHA. The results did, however, show some inhibition in comparison with untreated cells. Con A's inhibition did not exhibit a definite recovery period like PHA, but it resembled the untreated cells showing a slight increase in phagocytic activity due to aging. The inhibition of phagocytosis that did occur in Con A treated cells was presumed to be due to some other factor and not the mechanism under investigation.

To test the relationship between the two agglutinins as to their affinity to the monocyte receptors, Con A and PHA were added simultaneously to macrophage cultures. It was theorized that the phagocytic inhibiting property of PHA would act as a marker and if the property was shown, it could be assumed that PHA was adsorbed to the cells in the presence of Con A. If the inhibition was not observed it would indicate that Con A masked the effect of PHA. Our results showed that the PHA-Con A treated system acted very similar to the PHA control with a slightly shorter inhibitory period. Although this indicated that Con A did not mask the receptor sites of PHA it could not be shown that the reverse might be true ie., PHA masking Con A's receptor sites.

A detailed study of the agglutinin competition was made in which a series of systems were investigated allowing one agglutinin to adsorb to the cells for a period of time and then replacing it with the other agglutinin. By allowing Con A to adsorb first and then replacing it with PHA, we were able to confirm that Con A does not mask the ability of PHA to induce phagocytic inhibition of carbon. It was not possible to rule out the possibility of PHA masking the receptor sites for Con A since there were no definite markers showing the effect of Con A.

With the evidence presented in this report it is not possible to say that the inhibition of macrophage phagocytosis of carbon particles was a specific property of PHA. A more conclusive answer might be obtained if antilymphocyte globulin (ALG) were also used in comparison with PHA. Skoog and Lindahl-Kiessling (1970) did comparison studies with ALG and PHA in human lymphocyte cultures and found the properties of the two very similar. They showed that ALG added to cultures prior

to PHA masked the lymphocyte surface in such a way as to inhibit the PHA response, indicating that the cell receptor sites were shared by both types of antigens.

With the information gained from the phagocytic studies of PHA treated monocytes, we attempted to determine if vaccinia virus particles, like the colloidal carbon particles, were being inhibited from phagocytosis, thereby inhibiting the viral replication. To ensure that the inhibitory effect on viral replication was not due to a direct effect of the PHA on the virus, an experiment was performed involving only vaccinia and the agglutinin. Although concanavalin A has been shown to agglutinate certain arboviruses (Oram et al., 1971), we found no agglutination or any other effect of PHA or Con A to vaccinia.

To study the effect of PHA on phagocytosis of the virus, a short term experiment (24 hrs) was used as opposed to the long term experiments used previously. This would allow the study of the initial phagocytosis of the virus by PHA treated macrophages with the subsequent development of the viral life cycle. The short term experiment also allowed a more detailed assay of the virus in the initial infection.

In assuming that the inhibition of viral replication was due to the inability of the PHA treated macrophages to phagocytize the viral particles, it was expected that there would be a significantly higher number of viral particles detected in the supernatant of PHA treated cells than in either the control or Con A treated cells. This would have indicated that the viral particles in the PHA treated systems were not being phagocytized in the initial infection, but left free in the media. If the viral particles were not being phagocytized by the PHA treated monocytes,

then the cellular viral assay should have shown a lower number of viral particles than the untreated or Con A treated monocytes. This would have correlated with the supernatant assay in showing that the viral particles were being prevented from entering the cell.

Our results, however, did not correlate with the above assumption. There were no significant differences in the titers of the PHA treated cells with those of the control and Con A treated cells. Both the PHA and Con A treated macrophages apparently phagocytized the virus in a similar manner as the untreated cells. The lack of an increased titer in the supernatant and a decreased titer in the cellular portion indicated that PHA did not induce inhibition of phagocytosis of the viral particles. Even though our results showed that virus was being phagocytized by PHA treated macrophages (Fig. 13 and 14), they could not show inhibition of viral replication by PHA, because there was no viral replication in the control infection. It is possible that PHA has no observable effect on viral replication in the first 24 hours, as indicated by Figures 13 and 14. It seems likely that the time in which PHA actually begins inhibiting viral replication could be found by using similar, but slightly longer, experiments as those represented by Figures 13 and 14.

The possibility of PHA inducing an interferon-like substance capable of inhibiting vaccinia replication in the mononuclear host cells was eliminated as a source of viral inhibition after we demonstrated there was no difference in the viral titer whether the cells were treated with PHA for 90 minutes or 24 hours prior to infection. Wheelock (1965) reported that after 24 hours incubation of PHA with human

leucocytes, no viral inhibiting substance could be detected, although it could be detected at earlier incubation periods. Other workers (Friedman and Cooper, 1967) have reported similar results in lymphocyte studies. Duc-Nguyen and Henle (1966), however, could not detect any viral inhibitors in experiments similar to those of Wheelock (1965). Although an antiviral substance was not indicated in our results, confirmation should be made for biochemical testing of interferon production in PHA treated rabbit peritoneal macrophage cultures.

The mechanism of PHA's inhibiting effect on viral reproduction was not found in this investigation, however, a broader understanding of the action of PHA on rabbit peritoneal macrophages was attained. The ability of PHA to induce phagocytic inhibition in the peritoneal macrophages of carbon but not virus might be explained simply as the difference in an inert antigen and a living, infectious antigen or a more complex reaction may take place, such as PHA inducing a selective phagocytic inhibition by the cells.

Since the virus was shown to be phagocytized it seems likely that the viral inhibition results from some intracellular mechanism induced by PHA treatment. The possibility of PHA only acting on the cell surface and triggering certain intracellular events was suggested by Lindahl-Kiessling (1972). Besides an increase of DNA synthesis in PHA treated lymphocytes, other intracellular changes have been observed. Some of these changes are an increase in RNA synthesis (Mueller and Mahieu, 1966), increase in potassium uptake (Quastel and Kaplan, 1970), increase in protein synthesis (Hirschhorn _et al_, 1963), increase in lysozomal enzymes (Hirschhorn _et al_, 1967), and an increase in the

accumulation of glycogen (Quaglino *et al*, 1962). Even though most of these intracellular changes are involved in blast cell formation and mitosis of PHA treated lymphocytes, it is possible that some intracellular change in peritoneal macrophages may occur as a result of PHA attaching to the cell membrane, resulting in the inhibition of viral replication.

SUMMARY

The effect of phytohemagglutinin on vaccinia virus infection of rabbit peritoneal mononuclear phagocytes was investigated. It was found that vaccinia replication was inhibited by treatment of the phagocytes with PHA (25 ugm/ml).

Because of PHA's implications in cell membrane changes, a study was made to determine the phagocytic properties of PHA treated macrophages. PHA was found to inhibit phagocytosis of carbon particles by the macrophages. A direct relationship was observed between the length of PHA treatment of the cells and the length of time of phagocytic inhibition. The inhibition was found to be reversible whether the PHA treatment was for 90 minutes or for 8 days. The time of recovery from phagocytosis was also found to be dependent on the length of PHA treatment.

Concanavalin A was used as a comparative phytoagglutinin and was found to have very little effect in inhibiting phagocytosis. Simultaneous treatment with PHA and Con A revealed that Con A did not mask the ability of PHA to inhibit phagocytosis.

PHA did not affect the virus directly, but presumably affects the virus indirectly through the host cell. The inhibition of viral replication in the PHA treated host cell was shown not to be a result of the cells phagocytic inhibition produced by PHA, but more likely was due to some intracellular mechanism influenced by the PHA treatment.

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