AMERICAN UNIVERSITY OF BEIRUT

Thesis Title:

Phagocytosis of Poliovirus and Vaccinia Virus
by Mouse Peritoneal Macrophages

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Date of Thesis Presentation: April 19, 1969

31 OCT 1969

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May 30, 1969

PHAGOCYTOSIS OF POLIOVIRUS TYPE I AND VACCINIA VIRUS BY MOUSE PERITONEAL MACROPHAGES

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Submitted in partial fulfillment of the requirements
for the degree of Master of Sciences

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Beirut, Lebanon
1969

PHAGOCYTOSIS OF VIRUSES

ACKNOWLEDGEMENT

The writer is particularly indebted to Dr. G.A. Garabedian, Dr. J. Asmar, Dr. R. Matossian and Dr. F. Farah for their guidance, criticism and assistance throughout the preparation of this thesis.

To those who have assisted in various ways to make this work possible, the writer expresses his thanks and sincere appreciation.

ABSTRACT

Phagocytosis of poliovirus type 1 and vaccinia virus was measured quantitatively by a plaque assay method. The uptake rate of these viruses by the mouse peritoneal macrophages was rather low. Infective virions could be recovered inside the macrophages after 24 hours of incubation of the cells. No virucidal action of phagocytes could be demonstrated.

Although poliovirus type 1 and vaccinia virus were amply attracted to macrophages when brought in contact with these cells, but because of their superficial location, they were mostly neutralized by specific immune serum or eluted from the cell surface following repeated washing.

The attachment of these viruses to phagocytes was not influenced by the temperature of incubation. However, their penetration was temperature dependent. The uptake of virus was influenced by the concentration of the macrophages, but a mathematical relationship between the number of macrophages and virus particles and the amount of virus phagocytosed could not be established.

Experiments to determine the presence of specific receptors on macrophages for the attachment of poliovirus indicated that such receptors were absent.

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INTRODUCTION

The work of Metchnikoff, Almroth, Wright and others, at the turn of the century, has well established the defensive role of leucocytes and macrophages in bacterial infections. However, the role of leucocytes and macrophages in viral infections is less well defined. This is partly due to the fact that the polymorphonuclear leucocytes, which are of major importance in the phagocytosis of bacteria, appear to be of negligible importance in viral infections, although they have been shown to be capable of phagocytosing influenza virus (Boand et al., 1957).

Macrophages, however, seem to be important in certain generalized viral infections. It is possible that the fixed and wandering histiocytes remove virus particles from the blood (the kupffer cells of the liver and splenic macrophages being particularly important), from the serous cavities, the respiratory tract (alveolar macrophages) and the connective tissues. Depending on the nature of the virus, and the species of the host animal, these macrophages may either destroy the virus or support its multiplication, or be simple carriers of the virus. In many viral diseases (e.g. poxvirus infections) circulating monocytes act as vehicles for spreading the infection (Mims 1959). In other viral infections however, it is believed that phagocytosis

has no real bearing on the outcome of infections (Smorodintsev 1960). Nevertheless, the constant association of viruses with leucocytes and macrophages suggests that these cells may be important in some manner in the pathogenesis of viral diseases.

The experiments described in the present work were designed (1) to measure by means of a plaque assay the amount of active poliovirus type 1 and vaccinia virus particles phagocytosed in vitro by mouse peritoneal macrophages, (2) to determine the fate of the viral particles in the phagocytes during incubation of the phagocytes at various periods of time, (3) to determine the nature of the attachment of poliovirus type 1 to macrophages and, (4) to study the effect of various factors in virus-macrophage relationship.

REVIEW OF THE LITERATURE

Various methods have been used for the establishment of virus-leucocyte and virus-macrophage relationships and to determine the phagocytosis of viruses by leucocytes and macrophages. All the earlier experiments have used animal inoculation for the titration of the virus and these have determined only qualitatively the amount of virus phagocytosed by the leucocytes and macrophages.

The viremia of rinderpest was the first to be investigated. Schein (1917) demonstrated that the leucocytic fraction of blood was infectious whereas the virus was never associated with the erythrocytes. Ledingham in (1927), using scarification of rabbits for virus titration, showed that the local tissue macrophages play a great role in taking up vaccinia virus inoculated intracutaneously. He observed that the intracutaneous response to vaccinia in the rabbit is entirely modified in degree and character or completely abolished if the virus is inoculated in conjunction with india ink of sufficient concentration. He suggested that the mechanism of inhibition is due to the action of ink in stimulating the local reticulo-endothelial system to proliferate and thus enhance the defense forces of the animal. Smith (1929) confirmed the work of Ledingham and recovered vaccinia virus from the washed leucocytic fraction

of the blood of rabbits inoculated intravenously or intradermally. He recovered the virus at daily intervals from leucocytes despite the appearance of neutralizing antibodies. Long and Olitsky (1930) confirmed Smith's observation in indicating that in an immune animal the virus is removed from the circulation by the leucocytes and therefore is not free in the circulation to be neutralized by immune plasma. These results were also confirmed by Sabin (1935).

Todd (1928) demonstrated that in the centrifuged blood of infected fowls, the fowl plague virus is found mainly in the white cell layer. Hobbs (1928) suggested that myxoma virus was disseminated throughout the body within leucocytes and was not free in the blood stream. Fenner and Woodroofe (1953) showed that in rabbits infected with myxoma-virus, the virus in the blood was associated with white cells, probably with the lymphocytes.

Gresser and Chany (1963), employing primary cultures of human amnion cells, isolated measles virus from the washed leucocytic fraction of blood of 10 of 11 patients in the first 24 hours of exanthem and 1 of 8 in the second 24 hours of exanthem. The presence of the measles virus was determined by the appearance of the characteristic syncytial cytopathic effect (CPE) in cell cultures. Bedson and Duckworth (1963) found that rabbit pox virus in the blood was principally

associated with the "buffy coat" (leucocytes). They titrated the virus by inoculation of the chorio-allantoic membranes of 12- or 13-day-old chick embryos. Heggie and Robbins (1964) recovered rubella virus from leucocytes. The agent was detected by its capacity to interfere with subsequent infection with echovirus type 11 in monolayer cultures of primary African green monkeys.

Many of these early experiments also dealt with in vitro phagocytosis of viruses by leucocytes and macrophages. Douglas and Smith (1930) and Fairbrother (1933) found that vaccinia virus adsorbed to rabbit leucocytes in vitro, and was phagocytosed by these cells. Their experiments suggested strongly that phagocytosis is an important defense mechanism of immune animals against infection with vaccinia virus. Similar results were obtained in experiments with herpes simplex virus and rabbit leucocytes by Jamuni and Holden (1934). They reported that inactivation of virus occurred with immune cells in the presence of normal serum and suggested that leucocytes or possibly tissue cells may be even more important than the serum antibody in active herpes immunity. Sabin (1935) critically reviewed these studies and indicated that both normal and immune leucocytes exert a virucidal activity. He argued that the methods used were not adequate for making any conclusions. His experiments suggested that in the presence of immune serum the leucocytes

"fix" vaccinia virus but no evidence was obtained that it was subsequently destroyed. Also, the leucocytes of an immune rabbit were shown to contain active virus even 24 hours after inoculation.

Merling (1945), observed that intraleucocytic virus formed colonies inside the cell which survived the death of the leucocyte and continued to grow. Garabedian and Syverton (1955) demonstrated that herpes simplex virus adsorbed to rabbit peritoneal cells (neutrophils and macrophages) in vitro and suggested that it had probably been phagocytosed.

Smorodinstsev (1960) indicated that viruses, when freed from tissue fragments, do not undergo phagocytosis even in the presence of antibodies. He and his co-workers have used either virus attached to red cells or bits of infected tissue 3-7 μ in size. These were introduced into the peritoneal cavities of animals to produce leucocytic exudates. It was shown that after 4 to 6 hours of intraphagocytic residence the viruses were fully infectious at the time when tissue fragments were entirely digested.

Upon the development of fluorescent microscopy the individual infected cells could be identified with confidence in tissues and the process of infection could be analysed histologically. The use of fluorescent antibody techniques

has permitted identification of virus-infected cells in vivo and has markedly facilitated investigations dealing with pathogenesis of virus diseases. Fluorochromes have been employed in virology as immunofluorescent markers, cytochemical stains, mutagenic agents and as photodynamic compounds capable of inactivation of viruses. Coons et al. (1950) described for the first time a few simple experiments which demonstrated the specific staining of mumps virus. Coons and Kaplan (1950) improved the method by employing antibody labelled with fluorescein isocyanate as a histochemical stain. Liu and Coffin (1957) illustrated the usefulness of the fluorescent antibody technique in their studies of Canine distemper virus infection in ferrets and in dogs (Coffin and Liu 1957). On the third or fourth day after intranasal inoculation of ferrets, antigen was found in the leucocytes of the peripheral blood smears and by the sixth or seventh day blood smears were invariably positive. Mims (1959) showed that in mice infected with ectromelia virus, virus in the blood was largely associated with the blood cells, particularly platelets, lymphocytes and monocytes. Ectromelia virus was rapidly taken up by macrophages either in the peritoneum (Mims, 1964) skin (Roberts, 1962b) or by alveolar macrophages after aerosol infection (Roberts, 1962a). Employing fluorescent antibody staining technique, Roberts (1962a) established that one route of virus spread from the lung to the viscera was by the movement

of infected macrophages through the pulmonary lymph node. Roberts (1962b) also found that after scarification of virus into the dermis of mouse skin, the first infected dorsal cells were the macrophages.

Kovacs et al. (1963b) utilizing fluorescent antibody technique reported the presence of poliovirus type 3 antigen in leucocytes of peripheral blood smears of infected monkeys 10 days following the onset of paralysis. After inoculation of mice with purified fluorochrome labelled virus, bright green fluorescent dots of varying sizes were observed within leucocytes in blood smears prepared from peripheral blood of mice at different intervals. Maximum fluorescence appeared after 3 hours (Kovacs et al., 1963b).

Sommerville and Mac Farlane (1964) applied fluorescent antibody technique for the diagnosis of virus infections. They suggested that in the acute phase of virus infection the polymorphs carry virus antigenic material.

Boand, Kempf and Hanson (1957), using the fluorescent antibody technique of Coons and Kaplan, concluded that influenza virus could be phagocytosed by human and rabbit leukocytes in vitro. They suggested that the virus was first adsorbed on the leukocytes following which penetration into the cell occurred. The reaction of phagocytosis was greatly

enhanced by the presence of immune serum and when leukocytes from immune animals were used. These in vitro studies were elaborated further by Hanson et al. (1957). These workers injected large quantities of purified PR8 influenza virus intraperitoneally into normal and immune mice and sacrificed the animals at various intervals. By staining the peritoneal cells with a fluorescent dye they could show that virus was adsorbed rapidly to the mononuclear cells. Within 3 or 4 hours neutrophils appeared and by 16 hours the majority of the cells showed cytoplasmic fluorescence. Viral antigen persisted for 28 hours within the cells and infectious virus was easily recovered during this period. Inglot and Davenport (1962) showed that the leucocytes of peritoneal exudates appeared to contain a higher content of virus than blood obtained from infected mice. Phagocytosis of virus fluorescein labelled antibody complexes by leucocytes was clearly demonstrated. Johnson (1964a,b) demonstrated the uptake of herpes virus in vivo and in vitro by macrophages from mice inoculated intracutaneously, intraperitoneally and intranasally.

The effect of immune serum or immune cells on the uptake of virus and on the ultimate fate of virus still remains ill-defined. It is probable that in a number of instances the presence of immune serum does facilitate viral phagocytosis. In studying the blood clearance of P-32

Newcastle disease virus by the reticuloendothelial system in mice, Brunner and his co-workers (1960) found that treatment of the virus with antiserum increased its clearance. This may have been due to the larger aggregates of the virus-antibody complexes formed. Roberts (1964) showed that non-immune and immune mouse peritoneal macrophages phagocytosed ectromelia virus and immune cells were more actively phagocytic than non-immune cells. Ginder (1955) reported that immune serum and immune leucocytes and macrophages had a significantly greater neutralizing capacity for fibroma virus than immune serum or normal cells alone. Mims (1964) found that ectomelia virus-vaccinia immune serum complexes were taken up by liver macrophages, without reappearance of ectromelia antigen in macrophages of hepatic cells. This suggested that normally infectious virus particle was perhaps rendered digestible by the specific antiserum.

Phagocytosis of viruses was also studied by the use of electron microscopy. Nelson et al.(1963, 1964) using electron microscopy observed crystalline aggregates resembling columbia S-K virus within neutrophils in the brains of infected mice. Aronow et al. (1964) incubated rabbit peritoneal macrophages with T2 bacteriophages for 10-120 minutes. By electron microscopy they demonstrated adsorption of the phage to the cell membrane followed by incorporation of the virus within vacuoles but not within the cytoplasm. Neutrophils

also endocytosed the virus. Disintegration of T2 phage was not observed within macrophages, whereas destruction of the virus did occur within the cytoplasmic vacuoles of the neutrophils.

Much of the electron microscopy work was done with L and Hela cells. Vaccinia virus has been shown to be phagocytosed by L cells (Dales and Siminovitch 1961; Dales, 1963a) and fowlpox by chorioallantoic membrane (CAM) cells (Arhelger and Randall, 1964). Influenza virus was taken by the CAM and MK cells (Dales and Choppin, 1962) and NDV by culture cells (Mussgay and Weibel, 1962; Silverstein and Marcus, 1964). It was also, shown, by the use of electron microscopy that larger icosahedral particles entered cells intact within phagocytic vacuoles (Dales, 1962a; Holmes and Watson, 1963; Epstein et al., 1964; Dales et al., 1965). Particles of one of the small icosahedral agents of the papova group (Melnick 1962), have been observed inside cell vacuoles soon after inoculation of mice.

Many experiments were carried out in vitro to determine the possible multiplication of viruses in leucocytes and macrophages. Carrel (1925, 1926) inoculated plasma clot cultures of chicken leucocytes with Rous sarcoma virus. The virus multiplied (assayed by its oncogenecity for susceptible chickens) and in some instances the infected mononuclear leucocytes "rapidly acquired the characteristics of a culture

of macrophages from Rous sarcoma". Beard and Rous (1938) demonstrated the multiplication of vaccinia virus in cultured rabbit Kupffer cells and observed that after 7 days of cultivation the "cells appeared to be in excellent condition".

Florman and Enders (1942) observed that multiplication of vaccinia virus occurred in rabbit blood cells cultivated in roller tubes coated with plasma. Cells from both susceptible and immune rabbits supported the growth of the virus which continued at least for three weeks without causing "injurious effects". They postulated that mononuclear cells may not only disseminate virus, but perhaps harbor it for prolonged periods of time. Garabedian and Syverton (1955) also theorized that infected macrophages were responsible for latent infection with herpes simplex virus.

Newcastle disease virus was shown to multiply in chicken blood leucocytes (Bang and Warwick, 1957) and virulent strains caused complete cell destruction. The avirulent virus grew slowly and relatively poorly in macrophage cultures and destroyed the cultures only under special conditions. Goodman and Koprowski (1962a,b) demonstrated that macrophages from the spleen and peritoneal exudates of BS and $C3_H$ mice supported the growth of West Nile virus. Although virus multiplied for an extended period of time, no CPE was observed.

Berg and Rosenthal (1961) in their study of the growth of measles virus in leucocytes suggested that the neutrophils were unimportant for viral propagation. Employing a similar technique Berg (1961) was able to demonstrate propagation of echovirus 9 in suspensions of human leucocytes cultured from peripheral blood. Gresser and Chany (1964), however, were successful in demonstrating growth of both a virulent and an attenuated strain of poliovirus in preparations of human leucocytes. The behavior of poliovirus in this system differed in several respects from that observed in conventional monolayer cell cultures. In the usual cell cultures (Hela or KB cells, primary cultures of human amnion or rhesus monkey kidney cells, etc.) poliovirus multiplication begins within a few hours where as in leucocytic preparations there was a delay of 3-5 days. If virus was added on the fourth day of incubation, multiplication occurred without this initial lag period. These findings suggested that virus did not multiply in the cells originally present in the blood, but it did in cells either "changed" by cultivation or in their daughter cells. This impression was supported by the findings that even at the peak of viral multiplication only 4 percent of the cells were infected.

MATERIALS AND METHODS

Virus Strain

Poliovirus. Poliovirus type 1 attenuated Sabin Strain, kindly supplied in 1960 by Dr. T. Frothingham, Tulane University School of Medicine, New Orleans, was used throughout these experiments.

Vaccinia Virus. Lyophilized smallpox vaccine was obtained from the Lister Institute of Preventive Medicine, Elstree, Herts, England. The vaccine was diluted in growth medium (GM) and passaged twice on the CA membranes of 8-day-old chick embryos. The virus isolated from the pocks was passaged several times on MK cells.

Preparation of Polio and Vaccinia Stock Viruses

For stock virus preparation 0.5 ml of virus suspension was inoculated into bottles of MK monolayer cell cultures. The bottles were incubated at 37°C for 2 hours to allow virus adsorption on cells to occur. Maintenance medium (MM) was then added, and the bottles reincubated. After complete CPE had occurred, commonly within 24-48 hours, the virus suspension was harvested, centrifuged at 3000 revolutions per minute for 10 minutes to remove cell debris, tested for sterility and stored at -65°C until use.

Monkey Kidney Cell Cultures

The continuous MK cell line available in the Department

of Bacteriology and Virology, American University of Beirut, was kindly supplied by Dr. Chu of the Lebanese Government Laboratories at Fanar.

Phagocytes

The phagocytes were obtained from adult Swiss mice inoculated intraperitoneally with 3 ml of thioglycollate medium (Difco) three days prior to obtainment of cells. On the third day, the animals were killed by cervical dislocation, peritoneal cavities opened aseptically, and cells from each animal collected in GM containing penicillin 100 units/ml, and streptomycin 100 µg/ml. Heparin, 10 units/ml, was added to the GM during the collection of cells to prevent clumping.

The cells were washed twice with GM and the packed cells, obtained after the centrifugation of the suspension at 600 rpm for 5 minutes, were suspended in fresh GM and used in the experiments.

Growth Medium

Eagle's solution 10x	10 ml
New born calf serum	10 ml
Dextrose	0.6 gm

HBSS to bring the volume up to 100 ml.

A sufficient quantity of sodium bicarbonate (2.8%), was added to bring up the pH to 7.4. The medium was filtered

through Seitz filter and incubated for 72 hours at 37°C to test for sterility. Antibiotics were added just before use to have a final concentration of:

Penicillin ..... 100 units/ml

Streptomycin ..... 100 µgm/ml

Maintenance Medium

Lactalbumin hydrolysate ..... 0.5 gm

Dextrose ..... 0.3 gm

New born calf serum ..... 5 ml

HBSS to bring the volume up to 100 ml

A sufficient quantity of sodium bicarbonate (28%) was added to bring up the pH to 7.4. The medium was filtered through seitz filter and incubated for 72 hours at 37°C to test for sterility. Antibiotics were added just before use to have a final concentration of:

Penicillin ..... 100 units/ml

Streptomycin ..... 100 µgm/ml

Hanks's Balanced Salt Solution (Merchant et al., 1960)

Stock solution 20X

Solution A

NaCl ..... 80.0 gms

KCl ..... 4.0 gms

Na_2HPO_4 ..... 0.6 gm

KH_2PO_4 ..... 0.6 gm

$MgSO_4 \cdot 7H_2O$ ..... 2.0 gms

Dextrose ..... 10.0 gms

Triple distilled water to bring the volume up to 500 ml

Solution B

$CaCl_2$ ..... 1.4 gms

Triple distilled water to bring the volume up to 100 ml

Solution C

$NaHCO_3$ ..... 1.8 gms

Triple distilled water to bring the volume up to 100 ml.

Solutions A, B, C were kept separately in a refrigerator at 4°C.

HBSS Working Solution

Solution A ..... 5 ml

Solution B ($CaCl_2$ 1.4%) ..... 1 ml

Phenol red (0.4% ..... 0.25 ml

Triple distilled water to bring the volume up to 100 ml

The solution was dispensed in screw-capped bottles, and autoclaved at 120°C at 15 lbs pressure for 15 minutes. The pH was adjusted to 7.4 with 2.8 percent sodium bicarbonate.

Penicillin-Streptomycin Mixture

a) Penicillin G (potassium salt) ..... 1.000.000 units
Sterile triple distilled water ..... 10 ml
(Concentration of penicillin 100.000 units/ml)

b) Streptomycin ..... 1.000.000 µgm
Penicillin solution (100.000 units/ml), 10 ml

c) The suspension was distributed into small screw-capped tubes in 1 ml amounts and stored in a freezer at -20°C.

Phenol Red

Phenol red..... 0.8 gm
NaOH(N/20) ..... 57 ml
Triple distilled water to bring the volume up to 200 ml

The phenol red was dissolved in approximately 57 ml of N/20 NaOH, and the volume was brought up to 200 ml with triple distilled water. The color of the solution should be bright red. A purple color indicates an excess of NaOH and should be discarded.

The solution was sterilized in an autoclave at 15 lb pressure for 15 minutes, and stored in a refrigerator at 4°C in screw-capped tubes.

Eagle's TC Medium

Constituents for Eagle's medium were obtained in powder form in vials, as TC vitamins, TC amino acids, and TC glutamine 5 percent from Difco laboratories, Detroit, Michigan, U.S.A.

10 x Eagle's TC Medium

TC vitamins	0.1 gm
TC amino acids	0.22 gm
Glutamine 5% w/w	6.00 ml
HBSS solution to bring the volume up to	100 ml

The solution was dispensed in 10 ml screw-capped tubes and stored at -20°C until used.

Heparin Stock Solution

Heparin solution in GM (100 units/ml) was prepared from heparin injection obtained from Boots Pure Drug Co., Ltd. Nottingham England, and stored at -20°C until used.

Trypsin Stock Solution (50x)

Trypsin (Difco), 1:250, was diluted at 4°C in buffer solution to a 10 percent concentration. The solution was stirred with a magnetic bar for 10 minutes at 4°C. and was centrifuged at 10000 rpm for one hour at 4°C. The clear brownish supernate (50x concentrate) was filtered through a Seitz S-3 filter within 30 minutes. The filtrate was dispensed

aseptically in 10 ml amounts into screw-capped tubes and stored at -20°C.

Buffer Solution

NaCl	8.0 gms
KCl	0.4 gm
Na_2HPO_4	0.06 gm
KH_2PO_4	0.06 gm
Glucose	50 gms
Triple distilled water to bring the volume up to 1000 ml	

Trypsin Buffer 20x Stock Solution

NaCl	40.0 gms
KCl	2.0 gms
Na_2HPO_4	0.3 gm
KH_2PO_4	0.3 gm
Triple distilled water to bring the volume up to 250 ml	

For washing the cell monolayers the solution was diluted 20 x with triple distilled water and sterilized by filtration through Seitz filter and stored at 4°C in screw-capped bottles.

Trypsin 0.1 percent Working Solution

Trypsin buffer (20x)	5 ml
Concentrated trypsin (50x)	1 ml

Phenol red (0.4%) ..... 0.25 ml

Triple distilled water to bring the volume up to 100 ml

A sufficient quantity of sodium bicarbonate, 2.8 percent, was added to produce a pH of 7.4. The medium was filtered through Seitz filter and incubated for 72 hours at 37°C to test the sterility. Antibiotics were added just before use to have a final concentration of:

Penicillin ..... 100 units/ml

Streptomycin ..... 100 µgm/ml

New Born Calf Serum

New born calf serum was obtained from the Microbiological Associates Inc. Bethesda, Maryland. The serum was inactivated for 45 minutes at 60°C and stored at -20°C until use.

Agar-overlay Medium

Purified agar for tissue culture use was obtained from Difco laboratories, Detroit, Michigan. Agar .5gms was dissolved in 100 ml triple distilled water by boiling, dispensed in 10 ml amounts in screw-capped tubes and autoclaved at 15 lbs pressure for 15 minutes and stored at 4°C. Just before use the agar was melted by heating in a water bath. For agar overlay medium, 20 ml of 5 percent agar was mixed with 180 ml of GM.

Methyl Cellulose Overlay

Methyl cellulose (MC) 4000 centipoises (Dow Chemical Co., Midland, Michigan) was washed once with absolute ethyl alcohol, and air dried. Of this powder, 1.5 gm was suspended in 50 ml triple distilled water and placed in a water bath at 60°C for 30 minutes with occasional vigorous shaking. The suspension was then autoclaved at 120°C for 20 minutes and cooled to 45°C. Next, an equal amount of double strength GM, chilled to 4°C, was added to it and the mixture was stored at 4°C, at which temperature the MC was completely dissolved within 24 hours. Thereafter the overlay was either kept frozen at -20°C or stored at 4°C until used.

Preparation of Poliovirus Antiserum

Using undiluted tissue culture fluid as antigen, rabbits were injected intramuscularly at four sites on day 1 with 10 ml of a virus-adjuvant (Freund's) mixture. Virus culture fluid, 1 ml, was also given intravenously to each rabbit on days 1, 7, 14, 21 and 30. Finally, 10 ml of virus-adjuvant mixture were injected intramuscularly to each rabbit on day 21. On day 40, the rabbits were bled to death. The serum was separated, inactivated, sterilized by filtration through Seitz filter and stored in 10 ml amounts in screw-capped tubes at a temperature of -20°C.

Preparation of Vaccinia Antiserum

Adult rabbits were immunized by scarification of the

skin with vaccinia virus. When the skin was crusted, 3 weeks later, 10^6PFU ml of vaccinia virus was administered to each rabbit intravenously at weekly intervals for 4 weeks. The animals were bled to death 7 days after the last injection. The serum was separated, inactivated at 60°C in a water bath, sterilized by filtration through a Seitz filter and stored in 10 ml amounts in screw-capped tubes at a termperature of -20°C.

Glassware

The tissue culture bottles were obtained from Kimble Products, Owens, Illinois, U.S.A. Sterile screw-capped glass bottles of 200 ml capacity were used in all experiments unless stated otherwise. For plaque assays, sterile disposable tissue culture flasks (30 ml volume) were used. These were obtained from Falcon Plastics, Los Angeles, California.

Degree of Phagocytosis at a Fixed 60 Min. Period

To determine the degree of phagocytosis of polio type 1 and vaccinia viruses by mouse peritoneal cells during a fixed 60 minutes period, 0.5 ml of the virus suspension was added to 2.5 ml of a suspension of phagocytes in a small siliconized bottle with a broad base, and the mixture placed in an incubator at 35°-36°C for 60 minutes. At the end of this period the suspension was centrifuged at 600 rpm for 5 minutes, the cells were washed once with GM and the packed cells suspended in the original volume of GM. Next, to the virus-phagocyte suspension, was added an equal amount of dilute (1:4) anti-polio type 1 rabbit serum or undiluted anti-vaccinia rabbit serum, and the mixture was placed in an incubator at 35°-36°C for one hour. It was then removed from incubator, centrifuged at 600 rpm and the cells washed three times each time with 3 ml of GM. The cells from the third washing were suspended in 3 ml of GM, subjected to four cycles of alternate freezing and thawing and the resulting suspension plaque-assayed in MK cell cultures. An alternate experiment was run by adding 0.5 ml of the virus suspension to 2.5 ml of a suspension of phagocytes in a small siliconized bottle with a broad base, and the mixture, was stored at 2°-4°C for 60 minutes. Then it was processed as above.

For control purposes, the same amount of virus as used in the experiment, was diluted in 2.5 ml of GM, devoid of phagocyte, subjected to the same treatment as the virus-phagocyte suspension, and plaque-assayed. The same amount of virus diluted in 2.5 ml of GM devoid of phagocytes, treated as above but without exposure to antiserum was likewise plaque-assayed. The third washing from macrophage-virus mixture following treatment with antiserum, was tested for virus neutralizing capacity by neutralization test.

Determination of the Degree of Phagocytosis at Various Periods of Time

In order to determine the degree of phagocytosis of polio type 1 and vaccinia viruses by mouse peritoneal cells at various periods of time, a mixture of 2.5 ml phagocyte and 0.5 ml virus suspension was incubated at periods of 2, 4, 8, 16, 24 hours and plaque-assayed as described below. For control purposes, the same amount of active virus from a suspension containing the same proportion of virus with GM was plaque-assayed during the same intervals of time. Phagocyte counts on virus-phagocyte suspension were carried out concomitantly with these determinations.

Effect of Cell Concentration on Viral Phagocytosis

In order to observe the effect of cell concentration on viral phagocytosis, an experiment was carried out using

a constant amount of poliovirus type 1 together with various concentrations of mouse peritoneal cells. The procedure was the same as described above.

Effect of Repeated Washing of Cell-Virus Mixture on the Elution of Virus

Virus suspension, 0.5 ml, was added to 2.5 ml of suspension of phagocytes in a small siliconized bottle with a broad base and the mixture placed in an incubator at 35°-36°C for 60 minutes. At the end of this period the suspension was centrifuged at 600 rpm for 5 minutes, the cells washed repeatedly with GM and the packed cells suspended in the original volume of GM. Each washing was plaque-assayed on MK cell cultures.

Inactivation at 37°C of Polio Type 1 and Vaccinia Viruses

Polio type 1 and vaccinia viruses were maintained at 37°C in GM. Each day, 0.3 ml from each suspension was taken and plaque-assayed on monkey kidney cell cultures.

Determination of Viability of the Cells

To test the viability of mouse peritoneal cells, the trypan blue dye exclusion test was used. The cells before enumerations were mixed with a 0.2 percent solution of trypan blue. Those cells that absorbed the dye were considered as dead.

Enumeration of Cells

The mouse peritoneal cells were counted in an hemacytometer by the method described by Lennette and Schmidt (1966). The cells were dispersed by pipetting back and forth, treated with 0.2 percent of trypan blue and the chambers charged immediately. The cells in five large squares (each corner square and the center square) of both chambers were counted, omitting cells lying on the top and left hand lines but including cells lying on the bottom and right hand lines. The mean count of the 10 squares multiplied by 10,000 gave the number of cells per ml of solution.

Determination of the Phagocytic Activity of Cells

A mixture of $1x10^7$ mouse peritoneal macrophages and india ink (1:320 final concentration) was incubated at 37°C for 1 hour. A similar mixture was kept at 4°C during the same period of time. A drop of each was then examined microscopically to determine the percentage of cells that had phagocytosed carbon particles. About 50 percent of the phagocytes were found to have been loaded with carbon particles at 37°C while at 4°C only 25-35 percent of the cells engulfed carbon particles.

Receptor Assay for Poliovirus Type 1

Receptor assay for poliovirus type 1 virus was carried out according to a procedure described by Holland

and McLaren (1959). Mouse peritoneal macrophages, 7×10^6, were disrupted by 20 cycles of rapid freezing and thawing, and by thorough pipetting. Poliovirus type 1 was then mixed with untreated cell debris and with cell debris (a) treated with 0.25 percent trypsin for 15 minutes at 37°C, (b) heated at 60°C for 5 minutes, and incubated for 2 hours at 37°C. After this time the virus homogenate mixture was centrifuged and the sediment and the supernatant plaque-assayed on MK cell monolayers. Control virus titer was determined by incubating virus under the same conditions as above in the absence of cell debris.

Plaque Assays

1. Vaccinia. The methodology of the plaque-assay technique employed for counting vaccinia virus was that of Garabedian and Scott (1967) described for herpes simplex virus. Monolayers of continuous MK cells in rectangular screw-capped 30 ml capacity plastic bottles were washed with Hanks's BSS solution, pH 7, and inoculated with various dilutions, 10^{-1}-10^{-8}, of virus suspension. The bottles were incubated for two hours at 37°C, for adsorption of virus on cells to occur after which the inoculum was decanted and bottles covered each with 3 ml of methyl cellulose (MC) overlay medium. The cultures were reincubated until development of plaques occurred commonly within 4-5 days. At the end of this period, the MC was diluted out with saline (0.85%)

and decanted, the cells fixed with 95 percent methyl alcohol and stained with 0.5 percent methylene blue. Excess dye was rinsed off with tap water and plaques counted under the dissecting microscope.

2. Poliovirus Type 1. The methodology of the plaque assay employed for counting of polio type 1 virus was that of Holland and McLaren (1959) as modified by Soriano and Cherry (1966). Monolayers of continuous MK cells were prepared and the adsorption of virus was carried out as described. After removing the unattached virus, the cells were overlaid with 3 ml of MM containing 0.9 percent agar. The cell monolayers were reincubated in an inverted position until development of plaques occurred commonly within 48-72 hours. Formalin was added to the agar layer and allowed to act for 2 or 3 hours. Next, the firm gel overlay was separated from the cell sheet by vigorously hitting the opposite top side of the flask against the palm of the hand and holding the flask by the neck with the other hand. The cell sheet that was firmly fixed to the bottle as a result of formalin treatment was not affected by this procedure. After addition of tap water, the flask was shaken, the agar broken into small pieces and washed out with additional tap water. In some experiments, methyl alcohol was used as a fixative instead of formalin. The cell sheets were stained with crystal violet or methylene blue, and the plaques counted by the unaided visual inspection.

Phagocytosis* of Poliovirus Type 1 During 60-Minute Period at 37°C and 4°C

The results expressed in Table 1 are the average of 15 experiments. From this data it can be seen that 1.4×10^4 viral particles (PFU) were phagocytosed by mouse peritoneal macrophages at 37°C (column 1, Bb). It is evident from this Table (column 2, Bb) that only a minimum amount of active virus was detected in phagocytes when the virus-phagocyte suspension was incubated at 4°C. However, this value was increased approximately 100-fold when the incubation temperature was 37°C (column 1, 2, Bb and D). The ratio between the number of viral particles phagocytosed and the total number of phagocytes was estimated by dividing the number of phagocytosed active virus particles by the number of phagocytes used in the experiment and multiplied by 100 to obtain the percentage.

It is also evident from Table 1 that poliovirus type 1 particles, even at 4°C, did get attached to the phagocytes (column 1, 2, Aa), but apparently many of them were superficially located and were readily neutralized by the antiserum (column 1, 2, Ab). Thus, phagocyte-virus pellets from antiserum treated suspension contained less number of viral

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* The term phagocytosis, as used in this thesis, indicates the process in which the viral particles are taken up by the phagocytes beyond the reach of the neutralizing power of specific antiserum.

particles than those not treated with antiserum (column 1, 2, Ba, Bb). There was no great difference in the attachment of the virus at 4°C and 37°C (column 1, 2, Aa, Ba). The control virus suspensions were completely neutralized by the amount of antiserum used in the experiment (column 1, 2, D).

Phagocytosis of Vaccinia During
60-Minute Period at 37°C and 4°C

The results expressed in Table 2 are the average of 15 experiments. From the data of Table 2 (column 1, Bb) it can be seen that 1.2×10^3 vaccinia viral particles (PFU) were phagocytosed by mouse peritoneal macrophages when incubated at 37°C. This value decreased 240-fold when the temperature of incubation was brought down to 4°C (column 1, 2, Bb and D).

It is evident from Table 2 that vaccinia viral particles, even at 4°C get attached to the phagocytes (column 1, 2, Aa), but apparently, like poliovirus, many of them were superficially located and were readily neutralized by the antiserum (column 1, 2, Ab). The phagocyte-virus pellets from antiserum-treated suspensions contained less number of viral particles than those not treated with antiserum (column 1, 2, Ba, Bb). There was no great difference in the attachment of the virus at 4°C and 37°C (column 1, 2, Aa, Ba). The control virus suspensions were completely neutralized by the amount of antiserum used in the experiment.

Phagocytosis of Poliovirus Type 1
During Various Periods of Time

After 8 hours of incubation the amount of active poliovirus type 1 detected in phagocytes was increased by 7-fold, while the virus population remained constant in the mixture containing virus and GM (Table 3). After 24 hours of incubation there was a little decrease in the number of active virus detected in the phagocytes while the virus population decreased 10-fold in the virus-GM mixture.

After 8 hours of incubation at 37°C the number of phagocytes had declined from 6.5×10^6/ml to 5.2×10^6/ml of which 95 percent were viable as determined by trypan blue dye exclusion test. After 16 hours of incubation their number had declined to 3.4×10^6/ml of which 82 percent were viable. After 24 hours of incubation their number had further declined to 1.4×10^6 of which 76 percent were viable. It is possible that at this stage some of the cells were adherent to the glass surface of the container and were not brought into suspension despite repeated pipetting.

Phagocytosis of Vaccinia Virus
During Various Periods of Time

Table 4 shows that after 4 hours of incubation at 37°C the amount of active vaccinia virus detected in phagocytes was increased by 1.3-fold while there was no change in the virus

population of the virus-GM mixture.

After 16 hours of incubation the amount of active virus particles in the phagocytes increased 1.6-fold while the virus population decreased 1.5-fold in the virus-GM mixture. After 24 hours of incubation, there was a slight decrease in the amount of active virus particles in the phagocytes while the virus population decreased 2-folds in the virus-GM mixture.

After 4 hours of incubation at 37°C the number of phagocytes had declined from 6.5×10^6/ml to 6.2×10^6/ml of which 98 percent were viable as determined by trypan blue dye exlusion test. After 16 hours of incubation their number had declined to 3×10^6 of which 83.5 percent were viable. After 24 hours of incubation their number had further declined to 1×10^6 of which 74 percent were viable.

Action of Cellular Debris on Poliovirus Type 1

From the data in Table 5 it can be noted that poliovirus was not inactivated by the debris of mouse peritoneal macrophage. There were no differences in counts between the control virus and the virus treated with cell debris. Some virus was recovered in cell debris, but this may have resulted from enmeshment of virus in such debris not released by repeated washing. The virus enmeshed in cell debris, however, did produce plaques when assayed on MK cell monolayers.

It can also be noted from this Table that the amount of virus recovered was the same when the cell debris was untreated, treated with 0.25 percent trypsin, or heated at 60°C for 5 minutes, indicating that no specific receptor for poliovirus type 1 existed in mouse peritoneal macrophages.

The Effect of Concentration of Mouse Peritoneal Macrophages on the Uptake of Poliovirus Type 1

Experiments were run to determine the effect of concentration of macrophages on the uptake of poliovirus type 1. It can be seen from Table 6 that maximum amount of phagocytes was observed when macrophages in a concentration of 3×10^6 or 1×10^7 was used with 1×10^8 PFU/0.5 ml of virus. It can be concluded from these results that the concentration of mouse peritoneal macrophages may have some effect on the viral uptake as used in these experiments but no mathematical relationship can be established between the two.

Effect of Washing of Cells on the Elution of Poliovirus Type 1 and Vaccinia Virus From Mouse Peritoneal Macrophages

Figure 1 shows that poliovirus type 1 and vaccinia virus were eluted from phagocytes as a result of washing. It seems that some viral particles were loosely attached to the surface of the cells and were easily washed away while others were more firmly adherent or located deep in the cell wall and were not removed after repeated washing.

With the first washing of the macrophages 90 percent of the poliovirus and 92 percent of the vaccinia virus were released from the cells. It was noted that virus was present even in the tenth washing. This may have been due, at least partly, to breakage or lysis of cells during pipetting or other manipulation. The cell viability after the tenth washing was 50 percent of the original.

Inactivation of Poliovirus Type 1 and Vaccinia Virus at 37°C

Figure 2 shows the rate of inactivation of poliovirus type 1 and vaccinia virus at 37°C. It can be noted that both viruses were quite resistant to incubation at this temperature. During 6 days of incubation at 37°C poliovirus lost about 4 logs while vaccinia virus lost only 1.5 logs.

Table 1

PHAGOCYTOSIS OF POLIOVIRUS TYPE 1 DURING A 60-MINUTE PERIOD AT 37°C and 4°C*

		Temperature of incubation Column 1 37°C PFU/3ml	Column 2 4°C PFU/3ml
A. Virus-phagocyte** washed, suspended in GM, treated or not treated with antiserum and centrifuged	a. Supernate of "A" not treated with antiserum	1×10^5	2×10^4
	b. Supernate of "A" treated with antiserum	0	0
B. Virus-phagocyte pellet from "A" suspended in GM and subjected to 4 cycles of alternate freezing and thawing	a. Lysate from pellet not treated with antiserum	6×10^5	1×10^5
	b. Lysate from pellet treated with antiserum	1.4×10^4	5×10^2
C. Control. Virus treated with antiserum, subjected to same treatment as above in the absence of phagocyte pellet, subjected to 4 cycles of alternate freezing and thawing		0	0
D. Percent cells infected		0.21	0.0075

* The results expressed are the average of 15 experiments.

** Amounts used: virus, 1×10^8 PFU/0.5 ml; phagocytes, $6{,}5 \times 10^6$/2.5 ml.

Table 2

PHAGOCYTOSIS OF VACCINIA VIRUS DURING A 60-MINUTE PERIOD AT 37°C and 4°C*

		Temperature of Incubation Column 1	Column 2
		37°C PFU/3ml	4°C PFU/3ml
A. Virus-phagocyte** washed, suspended in GM treated or not treated with antiserum and centrifuged.	a. Supernate of "A" not treated with antiserum	1×10^4	3×10^3
	b. Supernate of "A" treated with antiserum	0	0
B. Virus-phagocyte pellet from "A" suspended in GM and subjected to 4 cycles of alternate freezing and thawing.	a. Lysate from pellet not treated with antiserum	2×10^4	5×10^3
	b. Lysate from pellet treated with antiserum	1.2×10^3	50
C. Control. Virus treated with antiserum, subjected to same treatment as above in the absence of phagocytes, pellet subjected to 4 cycles of alternate freezing and thawing.		0	0
D. Percent cells infected		0.018	0.0076

* The results expressed are the average of 15 experiments.

** Amounts used: virus, 3×10^6/0.5 ml; phagocytes, 6.5×10^6/2.5 ml.

Table 3

PHAGOCYTOSIS OF POLIOVIRUS TYPE 1 AT VARIOUS INTERVALS*

Time of Virus exposure to phagocytes; hours	Plaque counts/3 ml			Phagocyte Counts	
	Virus**-phagocyte***	Virus-GM	Virus-GM-antiserum	Total No/25ml	Percent viable
2	1.4×10^4	1×10^8	0	6.5×10^6	99
4	3×10^4	1×10^8	0	$6. \times 10^6$	97.5
8	1×10^5	1.8×10^8	0	5.2×10^6	95.2
16	1×10^5	1.5×10^7	0	3.4×10^6	82
24	7×10^4	1×10^7	0	1.4×10^6	76

* The results expressed are the average of 10 experiments.

** Original amount of virus added: 1×10^8 PFU/0.5 ml.

*** Original amount of phagocytes added 6.5×10^6/2.5 ml. Virus-phagocyte mixture was subjected to several cycles of alternate freezing and thawing after treating with antiserum.

Table 4

PHAGOCYTOSIS OF VACCINIA VIRUS AT VARIOUS INTERVALS*

Time of virus exposure to phagocytes; hours	Plaque counts/3ml			Phagocyte counts	
	Virus**-phagocyte***	Virus-GM	Virus-GM Antiserum	Total No/2.5ml	Percent viable
2	1.2×10^3	3×10^6	0	6.5×10^6	99
4	1.5×10^3	3×10^6	0	6.2×10^6	98
8	1.5×10^3	3×10^6	0	4.5×10^6	93
16	2×10^3	2×10^6	0	3×10^6	83.5
24	1.8×10^3	1.5×10^6	0	$.1 \times 10^6$	74

* The results expressed are the average of 10 experiments.

** Original amount of virus added: 3×10^6 PFU/0.5 ml.

*** Original amount of phagocytes added: 6.5×10^6/2.5 ml. Virus-phagocyte mixture was subjected to several cycles of alternate freezing and thawing after treating with antiserum.

Table 5

POLIOVIRUS TYPE 1 INACTIVATION BY VARIOUSLY-TREATED CELL DEBRIS OF MOUSE PERITONEAL MACROPHAGES*

Treatment	Supernatant PFU	Virus*** recovered from cell debris**** -PFU
A. Cell debris** untreated	98 x 10^4	70
B. Cell debris treated with 0.25 percent trypsin for 15 minutes at 37°C	9.85 x 10^4	50
C. Cell debris heated at 60°C for 5 minutes	9.95 x 10^4	20
D. Control. Virus-GM with no addition of cells	1 x 10^5	

* The results expressed are the average of 5 experiments.

** Cell debris was obtained by disruption of 7x10^6 mouse peritoneal macrophages.

*** Original amount of virus, 1x10^5 PFU.

**** For the determination of the virus recovered from cell debris, the debris was washed twice with GM then plaque-assayed.

Table 6

PHAGOCYTOSIS OF POLIOVIRUS TYPE 1 BY VARIOUS CONCENTRATIONS OF MOUSE PERITONEAL MACROPHAGES DURING A 60-MINUTE PERIOD*

Number of macrophages used/2.5 ml	1×10^6	3×10^6	4.5×10^6	10×10^6
Virus** recovered from phagocytes; PFU/3ml	2×10^3	7×10^3	1.5×10^4	1×10^4

* The results expressed are the average of 3 experiments.

** Original amount of virus: 1×10^8 PFU/0.5 ml.

DISCUSSION

The entrance of a viral particle into a living cell takes place according to two related biological systems. The first one of these recognizes the existence of receptors on the cell surface on which the virus is adsorbed; the viral nucleic acid subsequently gains entrance into the cell and directs the synthesis of a new virus (Holland 1964). The second system is based on the capacity of the cell to phygocytose or pinocytose the foreign particle or the virus by a process of engulfment (Dales 1965). There is evidence to indicate that upon attachment to host cells, which may involve specific membrane receptors, animal viruses including polio and vaccinia viruses, are transferred intact, with their genome still protected, into phagocytic vacuoles by an active and specific engulfment (Epstein et al., 1964; Fazekas de St. Growth 1948; Dales 1965).

Experiments reported herein show that the percentage of macrophages capable of phagocytosing viral particles is rather low (Tables 1 and 2). Observations similar to this have been made by Gresser and Chany (1964) in cultures of leucocytes and macrophages apparently susceptible to viruses. It must be noted, however, that the test procedure used in the experiments detected only the active viral particles. It is probable that only a few cells are able to incorporate virus or that viral particles are taken

up by the majority of macrophages but viral replication or release of virus is inhibited by an unknown mechanism in some or most of them. Not much is known about the intracellular events that follow phagocytosis. Gresser (1961) originally described the production of interferon by human leucocytes in response to viral infection. Glascow and Habel (1963), reported that leucocytes, predominantly of mononuclear type present in peritoneal exudates of mice, also produce interferon after infection with vaccinia virus.

The attachment of viruses to cell is not influenced by the temperature of the reaction mixture (Allison and Valentine 1960a). This was confirmed for poliovirus and monkey kidney cells by Batchtold et al. (1957). This also was shown by our experiments with poliovirus type 1 and vaccinia virus (Tables 1 and 2). The small difference in the attachment of the number of viruses observed at 4°C and 37°C may have been due to an increase in brownian movement of particles at higher temperatures. In other experiments, an increase in the number of polio type 1 and vaccinia viruses observed in the lysates of phagocytes following prolonged incubation of macrophage-virus mixtures at 37°C (Tables 3 and 4), may have resulted from adsorption of more virus on the phagocytes during this extended period of incubation. Apparently, at least a part of the virus was deeply located in the cell and so was unavailable for the

neutralizing action of immune serum. It is not possible to state at this time, however, if any multiplication of virus within the cell did occur during this period of incubation.

Although the attachment of poliovirus type 1 and vaccinia virus to mouse peritoneal macrophages appears not to be temperature dependent, our results indicate that the penetration of these two viruses into the cells is temperature dependent (Tables 1 and 2). This was also indicated for poliovirus by Holland and McLaren (1959). They demonstrated that all virus adsorbed to cells, is susceptible to homologuous antibodies at 1°C, but at 37°C a progressive decline in antibody sensitivity occurs. Therefore, it appears that the step following attachment is temperature dependent although studies pertaining to this subject are still incomplete.

It is then apparent that poliovirus type 1 and vaccinia virus do adhere to mouse peritoneal cells, but apparently their location on the cell is superficial as they are mostly neutralized when treated with specific immune serum. It is not certain, however, as to what proportion of these viral particles is engulfed by the phagocytes and inactivated, and what proportion remains outside the cell. In the animal body viral particles do get attached to

leucocytes, erythrocytes, platelets (Mims 1959) and possibly to tissue debris. In all these instances it is conceivable that cell or cell fragment associated virus is removed by the phagocytes, but under no circumstances the activity can be considered as being specifically directed toward the virus. Considering that the virus remains active within the phagocyte, as shown by results in Table 3 and 4, then it would be reasonable to assume that the entrance of cell fragment-free virus into the phagocyte does occur only occasionally and possibly is the result of a chance encounter between the virus and the phagocyte.

It is probable, that in vivo, viruses within leucocytes are protected from the action of specific antibody and non-specific viral inhibitors in the blood. Earlier investigators, Sabin (1935), Florman and Enders (1942) realized the importance of the protective role played by the leucocytes and suggested that viruses were disseminated to all tissues of the host by these cells. Whatever may be the case, it is conceivable that adult mouse peritoneal cells do not constitute an attractive site for poliovirus type 1 and vaccinia virus propagation and, certainly they should be unable to serve as vehicles for dissemination of viral particles into various tissues and organs of the animal. In all cases presence of virus within the leucocytes or macrophage does not necessarily indicate infection of the cell, since virus may persist within vacuoles and the process may even be reversible.

The phagocytosis of colloidal particles implies a primary attachment of particles to the cell surface and while this may be electrostatic in nature, specific site receptors may also be involved (Mims 1964). The importance of specific cell receptors in the infection of epithelial cells by myxoviruses is well astablished (Hirst 1943) and the importance of the role played by them in poliovirus infection has been pointed out (Holland and McLaren, 1959). It can be noted from our experiments (Table 5) that, mouse peritoneal macrophages do not contain specific cell receptors for poliovirus type 1. It can be further noted (Fig. 1) that after one hour of incubation, 10 percent of the virus was attached to cells but after 2 washings the percentage of virus remaining attached to these cells was only about 0.6. These results are in agreement with that reported by Holland et al. (1959) who found that about 1 percent of poliovirus inoculum was non-specifically bound to insusceptible cell surfaces. Holland (1964) further postulated that all susceptible human and MK cell cultures adsorbed poliovirus efficiently with first order kinetics where all non-primate insusceptible cells failed to adsorb detectable amounts of poliovirus in vitro. It should be pointed out that simple adsorption of virus on cell surface does not indicate infection of the cell. Darnell and Sawyer (1960) reported that a Hela cell clone, which was relatively resistant to poliovirus, adsorbed poliovirus and allowed penetration at the usual rate, but appeared to be relatively inefficient in releasing virus ribonucleic acids.

The plaque assay method of counting viral particles has the advantages over the electron microscopy and fluorescent microscopy that a quantitative relationship can be established between virus and cells. Excellent plaques were obtained with vaccinia virus when plaque-assay method described for herpes simplex virus by Garabedian and Scott (1960) were used in these experiments. Methyl cellulose overlay, used in these experiments, has the advantage of being stored at -20°C for long period of time and giving reproducible results. For poliovirus type 1 methyl alcohol fixation yielded better plaques than formalin fixation. Both for vaccinia and poliovirus, the plaques produced could be counted with a minimal amount of error.

In conclusion, consideration should be given to the fact that it would be difficult to extrapolate from an in vitro model, the events occurring within the infected animal. The literature concerning the relationship of viruses to various phagocytic cells has been extensively reviewed (Gresser and Lang, 1966). It is generally agreed that most viruses are phagocytosed by neutrophils and macrophages. This process, however, may not always be uniform. For example, some macrophages are genetically resistant to infection with certain viruses. In other instances, phagocytosis may take place but the cell remains uninfected or the virus gets inactivated after being phagocytosed even in

the presence of immune serum (Gresser and Lang, 1966). Despite numerous papers published about virus-phagocyte relationship, many of the results have been at variance or controversal because of the lack of uniformity of experimental conditions utilized by each investigator. Much more work is needed to establish the role played by leucocytes and macrophages in viral infections.

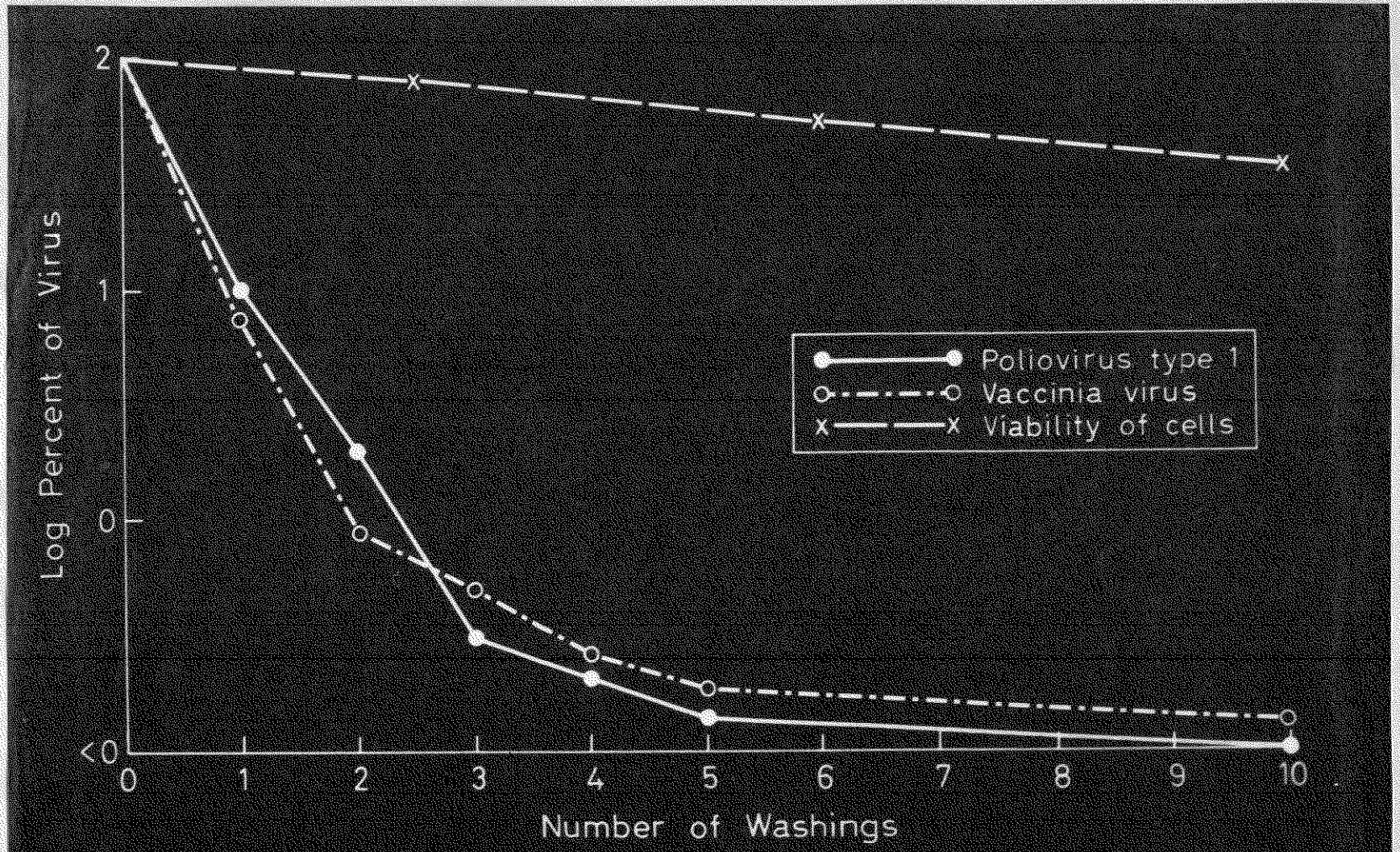

Fig. 1. The effect of repeated washing on the elution of poliovirus type 1 and vaccinia virus from mouse peritoneal macrophages.

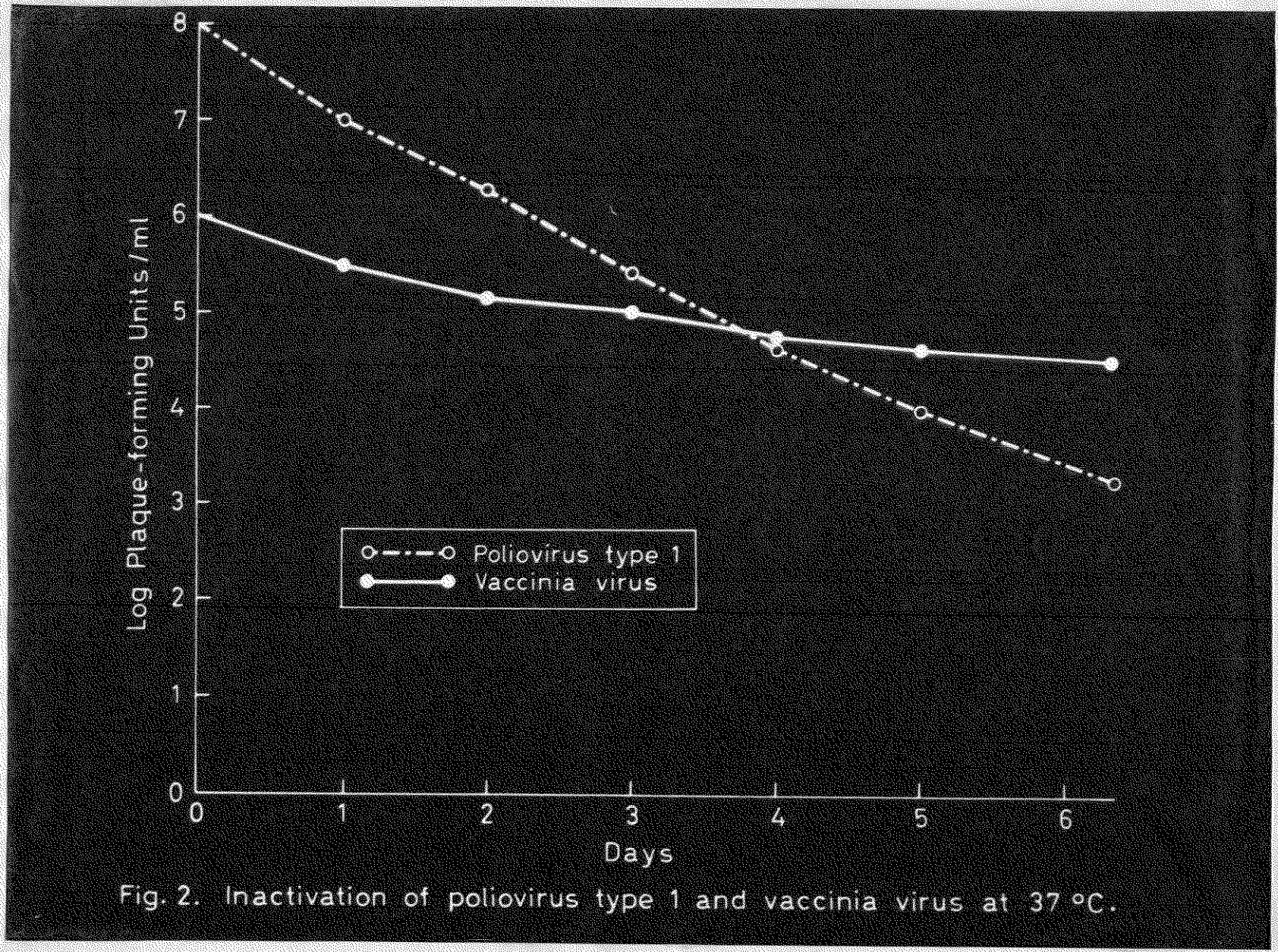

Fig. 2. Inactivation of poliovirus type 1 and vaccinia virus at 37 °C.

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