

FURTHER STUDIES ON THE INDUCTION OF IMMUNE RESPONSE  
WITH RIBONUCLEASE-SENSITIVE FRACTIONS OF IMMUNE MONOCYTES

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by

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## INTRODUCTION

It is common knowledge that animals, including man, develop immunity to antigens following an infectious or immunization process. This state of immunity means that the individual possesses mechanisms that make him specifically resistant to an infection. Changes have occurred in the animal after it has experienced the invasion of the infectious agent or its antigens. Immunologists have been concerned for many years with studies designed to describe the nature of the changes and to define the mechanisms responsible for the protection.

One mechanism of immunity is the formation of specific antibodies, first demonstrated by von Behring and Kitasato (1890). Antibodies are protein molecules, formed after antigenic stimulation, which will react specifically with the initiating antigen. Protective antibodies and certain other soluble substances are found in body fluids, particularly blood plasma and lymph.

However, in certain bacterial diseases such as tularemia, tuberculosis, undulant fever plus others which are characterized by the intracellular growth of the organism, antibodies cannot be shown to solely account for the protective mechanism of the immune host. From studies of the host-parasite relationship in these diseases has come the theory of cellular immunity.

Cellular immunity is usually thought of as a specific resistance against an infectious agent acquired by tissue cells typical of the reticulo-endothelial system whereby participation of specific antibody is not a requirement. The reticulo-endothelial system includes all the phagocytic cells of the body, except the circulating leukocytes.

Metchnikoff (1893) introduced the idea of cellular immunity some 80 years ago, when he recognized the crucial role of phagocytic cells as a host mechanism of defense against invading microorganisms. Before Metchnikoff presented his observations, it was generally held that phagocytic cells were simple scavengers whose function it was to pick up and carry to suitable disposal sites any foreign material which they chanced to encounter.

During the last 10 to 15 years more sophisticated research effort has been devoted to the factors which influence the function of the reticulo-endothelial system and the role of these cells in host protective mechanisms. Fong et. al. (1956, 1957) reported that animals immunized with BCG strain of tubercle bacillus developed a resistant population of histiocytes. They observed in vitro that the resistant histiocytes inhibited the intracellular growth of the organism. Normal histiocytes in vitro, infected identically, allowed intracellular multiplication of the organism followed by rapid cellular destruction.

However, although other investigators have reported similar results to those of Fong et. al. (1956, 1957), the cellular mechanism which is expressed in immune cells as inhibition of phagocytized organisms remains unknown.

In 1962, Fong et. al. contributed additional information regarding the phenomenon of cellular immunity. They demonstrated passive transfer of cellular immunity against Mycobacterium tuberculosis by the transfer of viable histiocytes from immune rabbits to normal recipient rabbits. Cellular immunity was observed in vitro as the ability of virulent tubercle bacilli infected immune histiocytes to resist intracellular multiplication and cellular destruction. This was not the case with normal cells, in which injurious destruction occurred rapidly. Water lysates of the immune histiocytes were found to pass the same state of immunity to normal recipients. Lysates of normal histiocytes resulted in no significant difference in the recipient's resistance.

Fong et. al. (1962) described the factor responsible for the immune response as one which could be transferred serially. This observation implied that the factor might be replicating and was not simple antigenic stimulation.

Further studies (Fong et. al., 1963) concerned with nature of the responsible factor showed it to be heat labile. The fact that it was heat labile and could replicate implied a nucleic acid as the responsible factor. Thus, immune

lysates were treated with ribonuclease or deoxyribonuclease prior to injection. Ribonuclease, but not deoxyribonuclease, inactivated the transfer factor. The mechanism involved was therefore an RNA-dependent phenomenon. Further investigation revealed the ribosomal RNA as the active factor of immune cell lysates. RNA extracts of immune histiocytes were found to be effective in the passive transfer of cellular immunity, but not as effective as the complete cell lysates. Fong et. al. (1963) presented convincing evidence, utilizing serological techniques, that the passive transfer of cellular immunity was not due to contaminating viable bacilli or bacillary antigens.

Mitsubishi and Saito (1962) demonstrated a successful in vitro transfer of cellular immunity against Salmonella enteritidis. Peritoneal cells were harvested from mice immunized with live S. enteritidis, planted, infected with heat killed S. enteritidis, and allowed to incubate for 24 hours. The supernatants were collected, filtered to remove the bacteria, and incubated with normal mouse cells for 72 hours. The supernatants were removed and the cultures were infected with live S. enteritidis. The cells treated with the supernatant from the immune cell system, inhibited intracellular multiplication, whereas cells treated with supernatants from normal cells demonstrated rapid cellular destruction.

Later studies (Saito and Mitsuhashi, 1965) described the active portion of the supernatant to be inactivated by ribonuclease but not with deoxyribonuclease. The transfer factor was non-dialyzable and could be inactivated in 24 hours at 37°C. The transfer agent was thought to be a type of cellular RNA that was released into the culture medium by immune macrophages upon exposure to the organism.

Further studies by Saito and Mitsuhashi (1965) showed that the in vitro transfer of cellular immunity to S. enteritidis was due to the ribosomal fraction of the immune monocytes originally cultured in the presence of killed organisms. The activity of the ribosomal fraction of these immune monocytes could be destroyed by treatment with ribonuclease but not deoxyribonuclease or trypsin. In vivo (Fong et. al., 1963) and in vitro (Saito and Mitsuhashi, 1965) studies coincided in their finding, in that the transfer agent appeared to be ribosomal RNA.

RNA's involvement in the induction of antibody has been widely accepted, but the exact function and relation to the antigen is yet to be clearly understood. Whether the active material is an RNA or an RNA-antigen complex, remains an open question. Even before the nucleic acid work of Fong et. al. (1962, 1963), Garvey and Campbell (1957) had shown that various antigens, labelled with radioactive isotopes, were stored in the liver tissue of the recipients in an immunogenic

form for several months.

Askonas and Rhodes (1964, 1965) studied the induction of antibody formation by RNA extracted from macrophages exposed to labelled antigens in vivo for different lengths of time. The extract obtained from the longer exposure was ten times more active than that obtained from the short exposure, although both were found to be immunogenic. Since they did receive immunogenic activity from the extract obtained from the short exposure, they concluded that an active process was necessary for RNA-antigen complex formation, but that the immunogenic response stimulated by the extracts was probably not due to the formation of specific informational RNA. It was thought that the RNA, complexed with the antigen, enhanced the uptake of the antigenic material by cells competent to produce the corresponding antibody molecules.

Friedman (1964) was able to demonstrate a specific induction of antibody, using RNA extracts of immune spleen cells, at 48 to 72 hours. The low molecular weight fraction of the RNA extracts was the active portion. Neither antigen nor antibody was found in the RNA extracts. He described three means by which the RNA extracts could have induced the antibody formation. They were: (1) an antibody coding mechanism; (2) an immunogenic antigen-nucleic acid complex; or (3) an antibody precursor. However, he did not state which of the three would best account for his results.

Fishman and Adler (1967) have presented new evidence regarding the role of macrophages and RNA in immune response. They have described the immunogenic RNA in terms of two active fractions. One of these fractions was macrophage-RNA linked to an antigenic fragment, with this fraction being responsible for a small molecular weight antibody formation. This material is probably similar to the RNA-antigen complex described in earlier work. In regard to the second fraction, they produced evidence showing that macrophages, possibly of a select type, produced a specific RNA in response to antigenic stimulation. Such RNA, free of detectable antigen and with an estimated molecular weight of  $1.6 \times 10^5$ , evoked antibody formation in lymph node cells.

The gap between antibody production and cellular immunity was bridged by the work of Kurashige et. al. (1967) and Mitsuhashi (1967). They demonstrated that monocytes from mice immunized with Salmonella enteriditis resisted cell degeneration and suppressed the intracellular multiplication of the organism in the absence of antibody in the cell culture medium. However, they found a cellular associated antibody that, when extracted from the cells, had inhibitory activity for S. enteriditis in the presence of complement and lysozyme. The antibody could be induced in vivo or in vitro with RNA extracts from immune cells. They suggested, on the basis of their results, that mononuclear phagocytes are



capable of antibody production.

Ostrander (1966) demonstrated suppression of intracellular multiplication of Francisella tularensis by cells from immune animals and immune cell lysate recipients. The passive immune macrophages were obtained from animals that had received an injection of a lysate of macrophages from immune rats 13 days prior to testing. He reported the transfer factor in the immune lysates to be RNAase sensitive, implying an RNA dependent phenomenon.

Stevenson (1967), also working with F. tularensis immune monocytes, demonstrated the presence of specific opsonins and later reported that they were also present in passive immune cell systems. In working with the RNAase-sensitive immune cell fraction, he described it as not having the ability to be serially transferred, and possibly being a species specific phenomenon.

The purpose of the work reported here was to further investigate the induction of immune responses of normal recipients receiving RNAase-sensitive fractions of immune cells.

## MATERIALS AND METHODS

### Bacteria

Two strains of Francisella tularensis, the attenuated strain, Jap, and the virulent strain, Schu, were used in this study. These two strains were obtained from the Communicable Disease Center, Atlanta, Georgia. Both strains were cultured on glucose cysteine blood agar (GCBA) (Downs et. al., 1947), 24 hours prior to experimentation. Virulence of Schu was maintained by routine passage through laboratory rats.

### Preparation of a Standard Bacterial Suspension

A 24 hour culture of bacteria was grown on GCBA, suspended in 0.85% saline, transferred to a sterile spectrophotometer tube, and adjusted to 20% transmission at a wave length of 600 mu on the Spectronic-20. Serial 10 fold dilutions were made and plated onto GCBA, incubated 48 to 72 hours, and the colonies counted to determine the precise viable count.

### Experimental Animals

The rats used in this study were mixed breeds of black, albino, and hooded animals originally obtained from the Vlk Animal Farm, Marion, Kansas, and from the Lemberger Company, Oshkosk, Wisconsin. The parental stock of the

white mice used were obtained from Kansas State University, Manhattan, Kansas. Healthy young adults were used in all experimentation.

### Immunization Procedure

Live and killed antigens were employed for immunization purposes. Rats were immunized by injecting one ml of  $10^6$  live Jap organisms per ml, intraperitoneally (I.P.), and challenged in three weeks with  $10^7$  Schu per ml. Animals surviving after three weeks were considered immune.

Adult mice were immunized with 0.5 ml of live Jap, containing  $5 \times 10^3$  organisms per ml, I.P., and challenged in four weeks with 0.5 ml of Jap, diluted to  $5 \times 10^7$  organisms per ml, I.P.

Killed antigen immunization consisted of one intraperitoneal injection; rats received one ml and mice 0.5 ml of the bacterial suspension diluted to  $10^6$  organisms per ml. The antigen was killed in 2% formalin for 24 to 48 hours, washed twice in 0.85% saline, and plated for viable organisms.

### Agglutination Test

Blood was collected from the hepatic portal vein of rats and mice, pooled, allowed to clot, and the serum collected.

A 24 hour culture of F. tularensis, Jap strain, was suspended in 0.85% saline, washed once and adjusted to a standard suspension.

Serial two fold dilutions, ranging from 1:2 to 1:256, were made of the serum to be titered. Equal volumes of antigen and diluted serum were mixed, incubated for three hours at 37°C, placed in the cold overnight, and read.

### Glassware

All glassware was soaked a minimum of 24 hours in a dilute Alconox solution containing 10 ml of Clorox per liter of solution. The glassware was then washed in Alconox, rinsed five times in tap water, eight times in distilled de-ionized water, and allowed to air dry.

Pipettes and flying coverslips were exceptions to the above procedure. Pipettes were soaked in a strong Clorox solution for a minimum of 24 hours, placed in a pipette washer for three to four hours, rinsed individually in distilled de-ionized water, and allowed to air dry.

The flying coverslips were washed in a dilute solution of Alconox with constant gentle agitation for 24 hours, and allowed to rinse in tap water for another 24 hours. They were then rinsed individually ten times in four consecutive distilled de-ionized water baths and one 95% ethanol bath. The coverslips were stored in 95% ethanol until autoclaved, and used.

### Tissue Culture Medium

The cell culture medium used in this study was Scherer's containing heparin, 1:10,000, 100 units of penicillin per ml and 30% (v/v) heated calf serum. Serum was omitted from the opsonic study because of its ability to enhance phagocytosis.

The calf serum was prepared in this laboratory using blood from fasting, freshly slaughtered young animals at the Fanestil Packing Co., Inc., Emporia, Kansas. After the blood clotted, the serum was decanted into glass tubes, centrifuged at 1000 xg for 10 minutes, bacteriologically filtered, aliquoted and immediately frozen. Before use, the serum was heat inactivated at 56°C for 30 minutes.

### Harvesting of Peritoneal Macrophages

The animals were sacrificed with chloroform, and immediately secured to a 12" x 12" board. The skin was excised aseptically from the ventral side, exposing the abdominal muscles. Seven to 10 ml of harvesting media was injected into the peritoneal cavity, and the walls massaged to suspend the macrophages. Cellular exudate was collected with a capillary pipette through an incision made in the peritoneal wall. Like exudates were pooled, counted, and standardized with Scherer's, serum, and the attenuated

strain, Jap. One ml aliquots were delivered into flying coverslip tubes and incubated at 37°C.

The pH was adjusted with 0.5% NaHCO<sub>3</sub>, in Scherer's solution delivered through a 25 gauge needle.

### Opsonic Study

Peritoneal macrophages were harvested, counted, and adjusted with Scherer's solution to  $3 \times 10^6$  per ml. The cell suspension was infected with F. tularensis, Jap strain, at a ratio of five bacteria per cell, planted in flying coverslip tubes and allowed to incubate for three hours at 37°C. For fluorescent staining, duplicate coverslips were placed in acetone for five minutes, allowed to air dry, placed in a moisture chamber, flooded with the adsorbed conjugate and stained 30 minutes at 37°C. The coverslips were removed from the moisture chamber, washed in three changes of 0.02 M phosphate buffered saline (PBS), pH 7.2, and allowed to remain in the last bath 15 minutes. They were mounted on microscope slides with buffered glycerol (pH 8.0) and observed through an ultra-violet microscope.

Three selected areas were counted on each coverslip, counting 100 cells per area, scoring each cell that contained one or more organisms (Plate 1 and 2). The average was calculated per coverslip, then the average of the two coverslips was recorded as the per cent phagocytosis of that particular system.

### Viable Count Studies

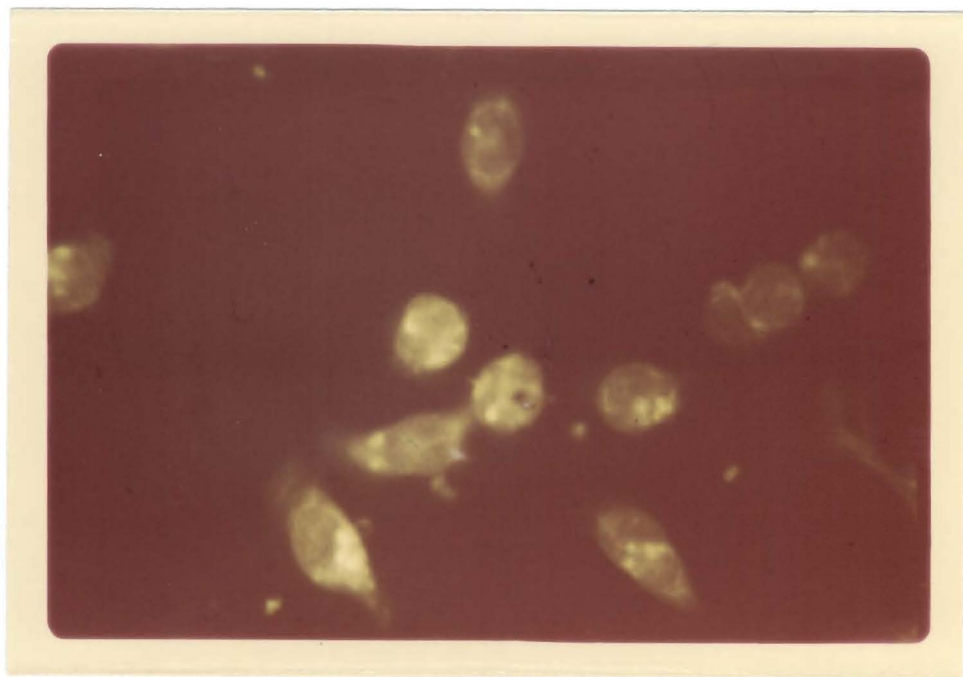
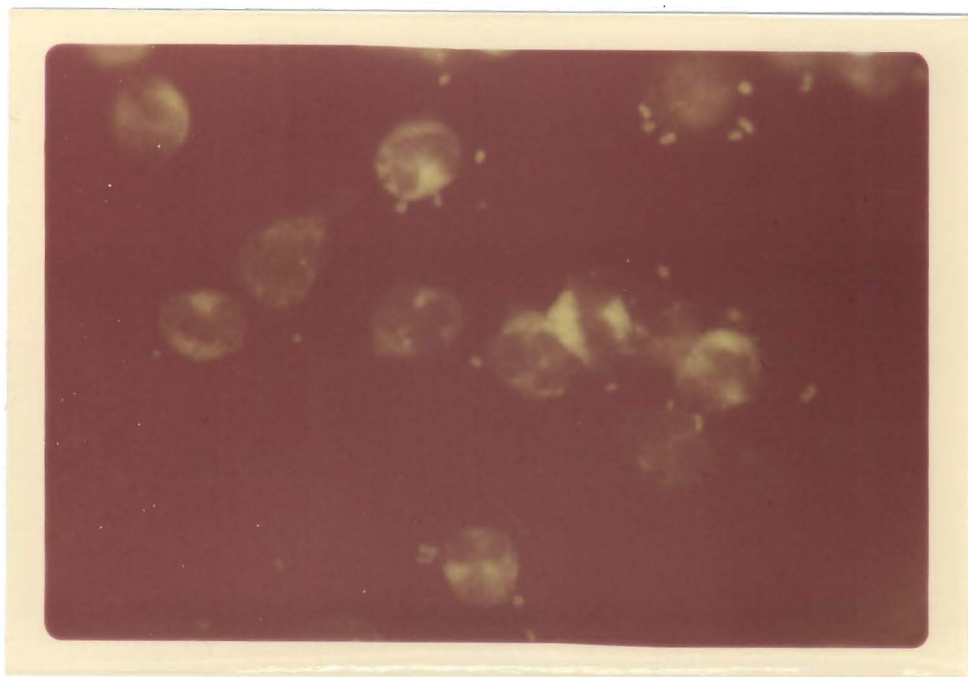
The peritoneal macrophages of the animals to be studied were harvested, counted, and adjusted to  $3 \times 10^6$  cells per ml with Scherer's, heated calf serum, and the inoculum of F. tularensis, Jap strain. The inoculating dose was  $10^4$  organisms per ml. The infected cells were planted in one ml aliquots into flying coverslip tubes. The study was followed by plating onto GCBA, and staining with May-Grunwald Giemsa 0, 24, 48, 72, and 96 hours.

At described times, a representative coverslip from each system was crushed and thoroughly ground, thereby disrupting the cells and releasing the bacteria into the media. The mechanical disruption was done with a glass rod formed to fit the bottom of the flying coverslip tube. Serial 10 fold dilutions were made of the media in saline, and 0.1 ml quantities of appropriate dilutions were plated onto duplicate GCBA plates. The inoculum was spread over the surface of the GCBA, the plates inverted and incubated at 37°C for 48 to 72 hours, and counted. Viable plate counts were considered to indicate viable intracellular organisms since earlier studies had shown that F. tularensis did not survive in cell culture media alone (Ostrander, 1966).

Plate 1: Fluorescent-antibody stain of F. tularensis infected "non-immune" rat peritoneal mononuclear cells (600X).

Plate 2: Fluorescent-antibody stain of F. tularensis infected "immune" rat peritoneal mononuclear cells (600X).





Representative coverslips from each experimental system were removed from the tubes and allowed to air dry. The coverslips were then placed in the fixative, methanol, for five minutes, stained with May-Grunwald for two and one-half minutes and Giemsa for four minutes, rinsed in acetone for one minute, rinsed again for one minute in acetone-xylene (1:1), and cleared in xylene for 10 minutes. The stained coverslips were attached to a microscope slide, cell side down, with permount and observed.

#### Lysate Preparation

Cell lysates were prepared by disrupting cells with water, freeze-thaw, and sonication. The water lysis method, as described by Fong et. al. (1962), was the procedure most consistently used in this study. Peritoneal macrophages were harvested from immune animals in Hanks' balanced salt solution containing heparin and penicillin. The cells were washed three times in Hanks' and counted after the second wash. After the third wash the cells were resuspended in distilled de-ionized water to a concentration of  $10^7$  cells per ml and placed in a water bath at  $37^{\circ}\text{C}$  for three hours with occasional agitation. The lysate was centrifuged at 2100 xg for 10 minutes, the supernatant decanted and injected intraperitoneally into normal animals. Rats and mice received injections of two and one ml respectively. The

lysate was plated to check for viable organisms.

The freeze-thaw method of lysis consisted of freezing and thawing the cell suspension three times. Lysis by sonication was accomplished with a sonifier at a setting of seven amperes for four one minute periods with a one minute interval between sonication periods.

#### RNAase Treatment of Lysate

Enzyme treatment of lysate was done using beef pancreatic ribonuclease, five times crystalized, from Miles laboratories, Elkhart, Indiana. The enzyme was dissolved in 0.02 M PBS, pH 7.2, and sterilized by filtration. The enzyme solution was heated at 80°C for 10 minutes to remove contaminating enzyme activity (Fishman and Adler, 1963). A sufficient amount of the RNAase solution was added to the lysate to give a concentration of: 1 mg, 0.5 mg, and 0.1 mg per ml of lysate. The enzyme treated lysate was incubated at 37°C for 20 minutes and injected into the normal recipients.

#### Fluorescent Antibody Preparation

Fluorescent antibody was prepared according to Rinderknecht (1962), but using gamma globulin instead of whole serum (Cherry, 1960). A rabbit was immunized with multiple injections of formalin killed F. tularensis, Jap strain. Blood was taken from the immune animal 10 days after

the last injection, clotted, and the serum collected. The globulins were precipitated with saturated ammonium sulfate overnight at 5°C, the mixture centrifuged, the supernatant discarded, and the pellet re-dissolved in distilled de-ionized water equal to the original volume of serum. The globulins were re-precipitated, centrifuged, and re-dissolved until the wash was free of hemoglobin. The pellet of the final wash was re-dissolved in distilled de-ionized water,  $\frac{1}{4}$  the original volume of serum, and dialyzed against 0.85% saline, at 5°C until a mixture (1:1) of saturated  $\text{BaCl}_2$  and the dialyzing solution gave no precipitate. The protein content of the conjugate was determined by the Biuret method and adjusted to 2% with saline

A mixture of one part conjugated globulin and one part 0.05 M sodium carbonate-bicarbonate buffer of pH 8.5 was shaken for approximately three minutes with  $1\frac{1}{2}$  mg of "celite" (a powder consisting of 10% fluorescent dye dispersed on diatomaceous earth or other inert materials) per ml of mixture. The mixture was centrifuged at 800 xg for three minutes and the supernatant allowed to flow into a column (1.5 x 18 cm) of "Sephadex G25" (Pharmacia Uppsala, Sweden). The Sephadex column was prepared in advance by pouring a slurry of Sephadex into a column, allowing the gel to settle, followed by washing with several column volumes of 0.02 M sodium phosphate buffer of pH 6.5. The labelled protein was

collected with an increase in volume of 50 to 100 per cent. The eluted fluorescent protein was aliquoted into one ml quantities and kept frozen until needed.

When ready to use, a needed volume of labelled conjugate was thawed and adsorbed for 30 minutes at 5°C with 100 mg anhydrous rat liver powder per ml of conjugate (Cherry, 1960). The adsorption of the liver powder was necessary to eliminate non-specific staining components. The adsorbing conjugate was centrifuged for 10 minutes at 2100 xg and the supernatant drawn off ready for fluorescent staining.

## RESULTS

### Ribonuclease Sensitivity of Immune Cell Lysate

Ostrander (1966) reported and Stevenson (1967) confirmed that the transfer of resistance by immune cell fractions was ribonuclease, but not deoxyribonuclease, sensitive. The specific destruction of the transfer phenomenon by the ribonuclease was questionable because of the concentration used. Therefore, immune cell water lysates were prepared and divided into four portions. One portion, the control, remained untreated, the other three were treated with either 0.1 mg, 0.5 mg, or 1.0 mg RNAase per ml of lysate. The lysates were then injected intraperitoneally into normal rats. After 14 days, cell suspensions were obtained from the recipient animals, and the protection quantitated by phagocytic and viable count studies. Cell systems derived from recipients of ribonuclease treated immune lysates failed to demonstrate any protection as compared to cells derived from the untreated immune lysate recipients (Figure 1 and 2). Therefore, it was concluded that the transfer of resistance to F. tularensis by immune cell fractions was mediated by a ribonuclease sensitive mechanism.

The fact that the transfer phenomenon was an RNAase sensitive phenomenon was also demonstrated in mice using 0.1

Figure 1: Lack of opsonin induction by immune cell lysates treated with different concentrations of RNAase prior to injection into normal recipients.

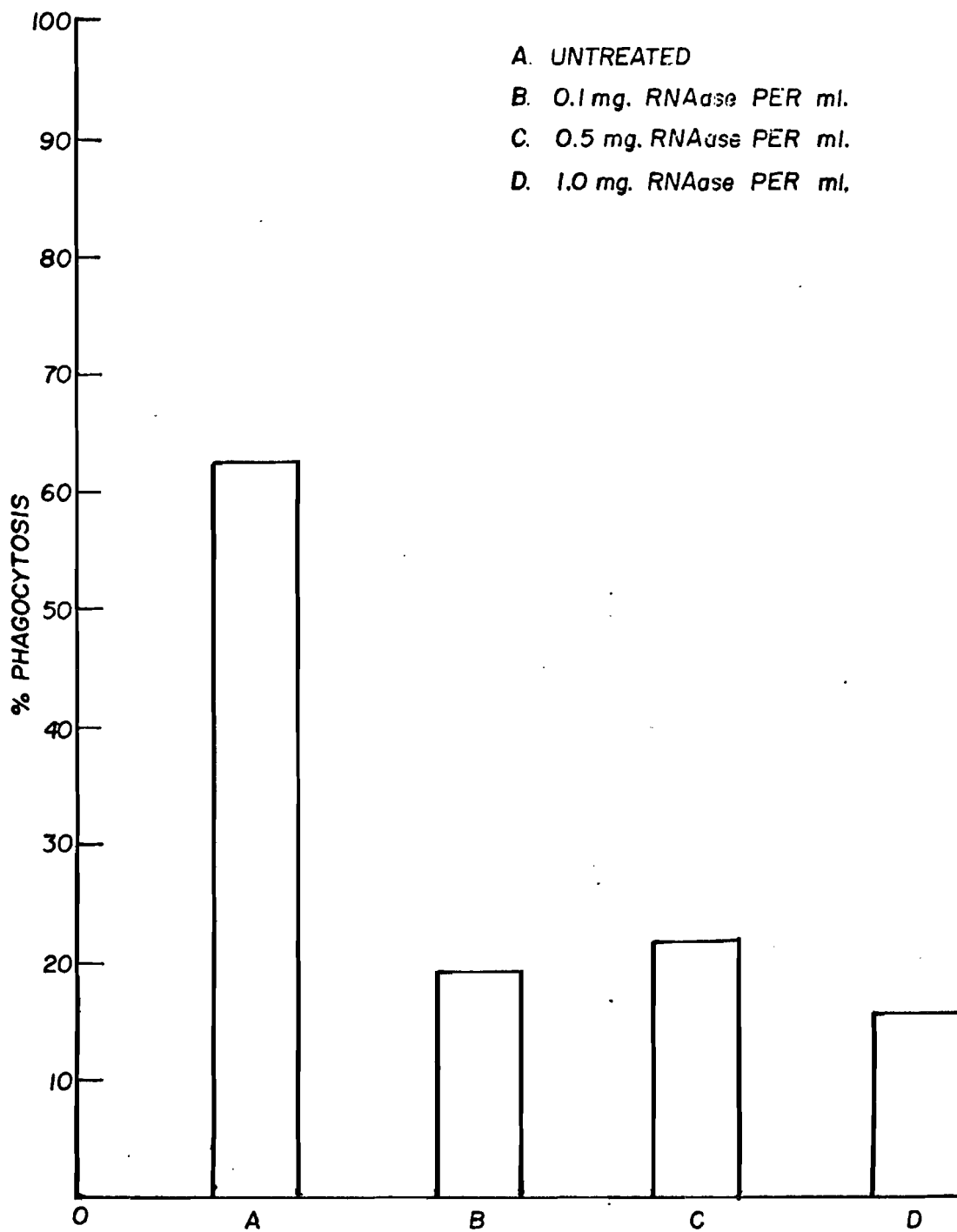
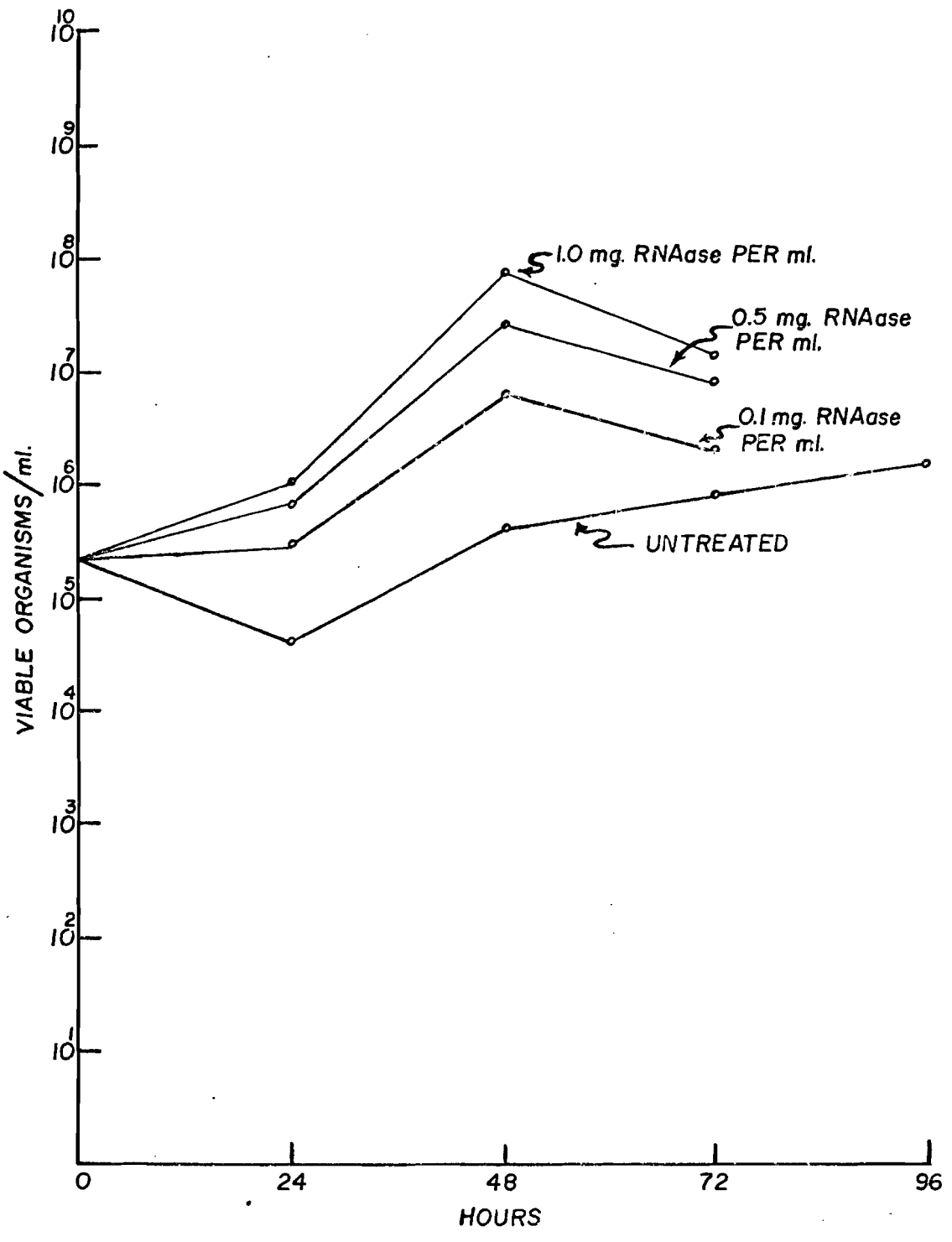




Figure 2: The multiplication of F. tularensis in mononuclear cell cultures prepared from recipients of immune cell lysates treated with different concentrations of RNAase prior to injection.



mg RNAase/ml of lysate (Figure 3). The lysate was obtained from killed antigen immune mouse cells.

Live Opposed to Killed Antigen Immunization in Cellular Protection.

A clue to the mechanism of cellular immunity was thought to possibly evolve from studies of killed and live antigen immune cells. In vivo, live antigen immunization protects the host from multiplication of the virulent strain of the particular organism, but the same does not hold true for killed antigen immune animals. Killed antigen immunized animals produce antibodies that can be demonstrated in vitro by seriological techniques, but the product is not highly protective in vivo.

Animals were immunized with live and killed F. tularensis, their cells harvested, and the cellular protection quantitated by phagocytic and viable count studies. The phagocytic activity was almost identical for the two cell systems (Figure 4). However, the viable count study demonstrated that the killed antigen immune cells suppressed intracellular multiplication whereas the live antigen immune cell only inhibited it, resulting in a dramatic difference between the two systems (Figure 5).

Live and killed antigen immune cell water lysates were prepared and injected intraperitoneally into normal animals.

Figure 3: Lack of opsonin induction by immune cell lysate treated with 0.1 mg RNAase/ml prior to injection into normal recipients.

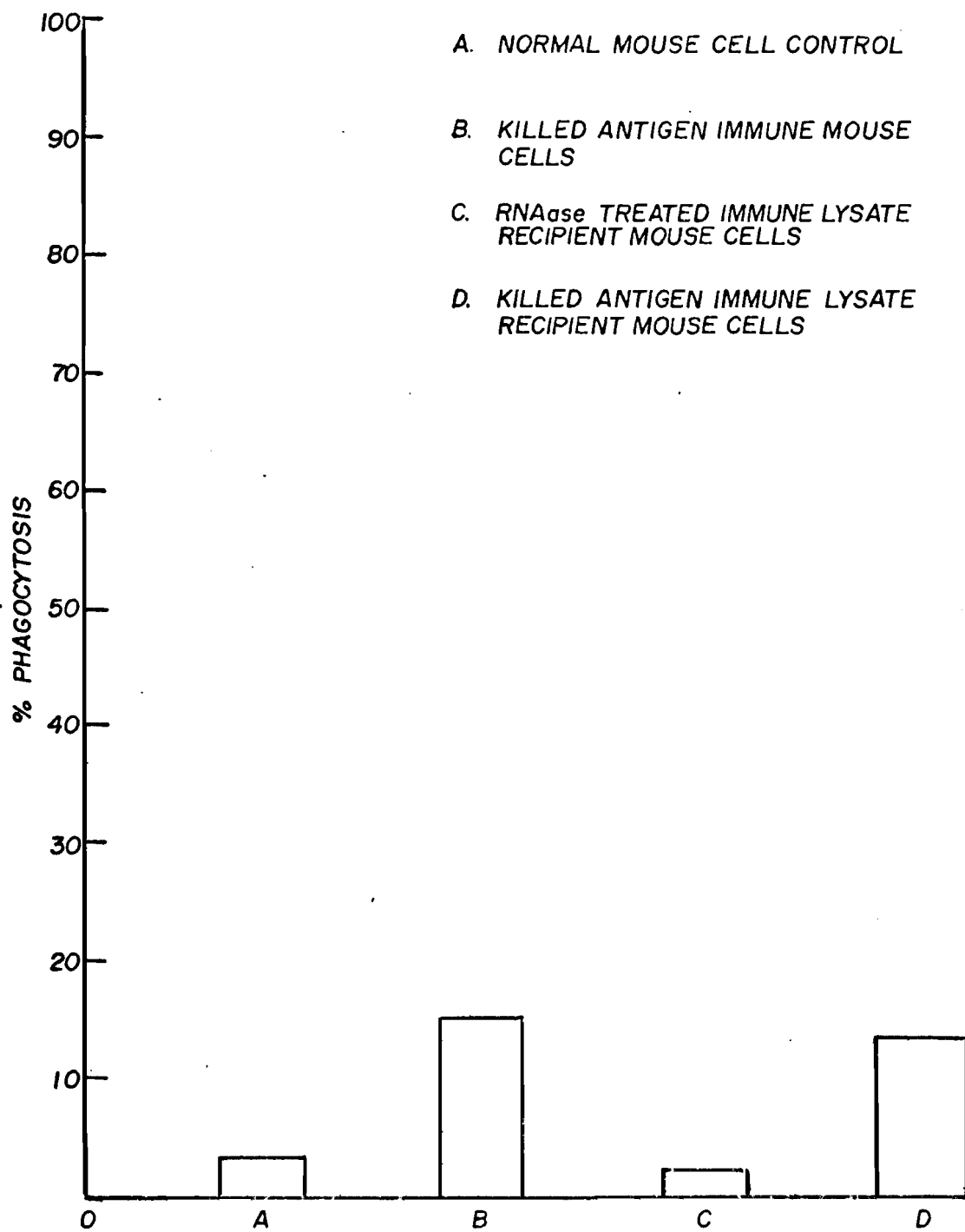


Figure 4: Induction of opsonin activity in normal rats immunized with live and dead F. tularensis.

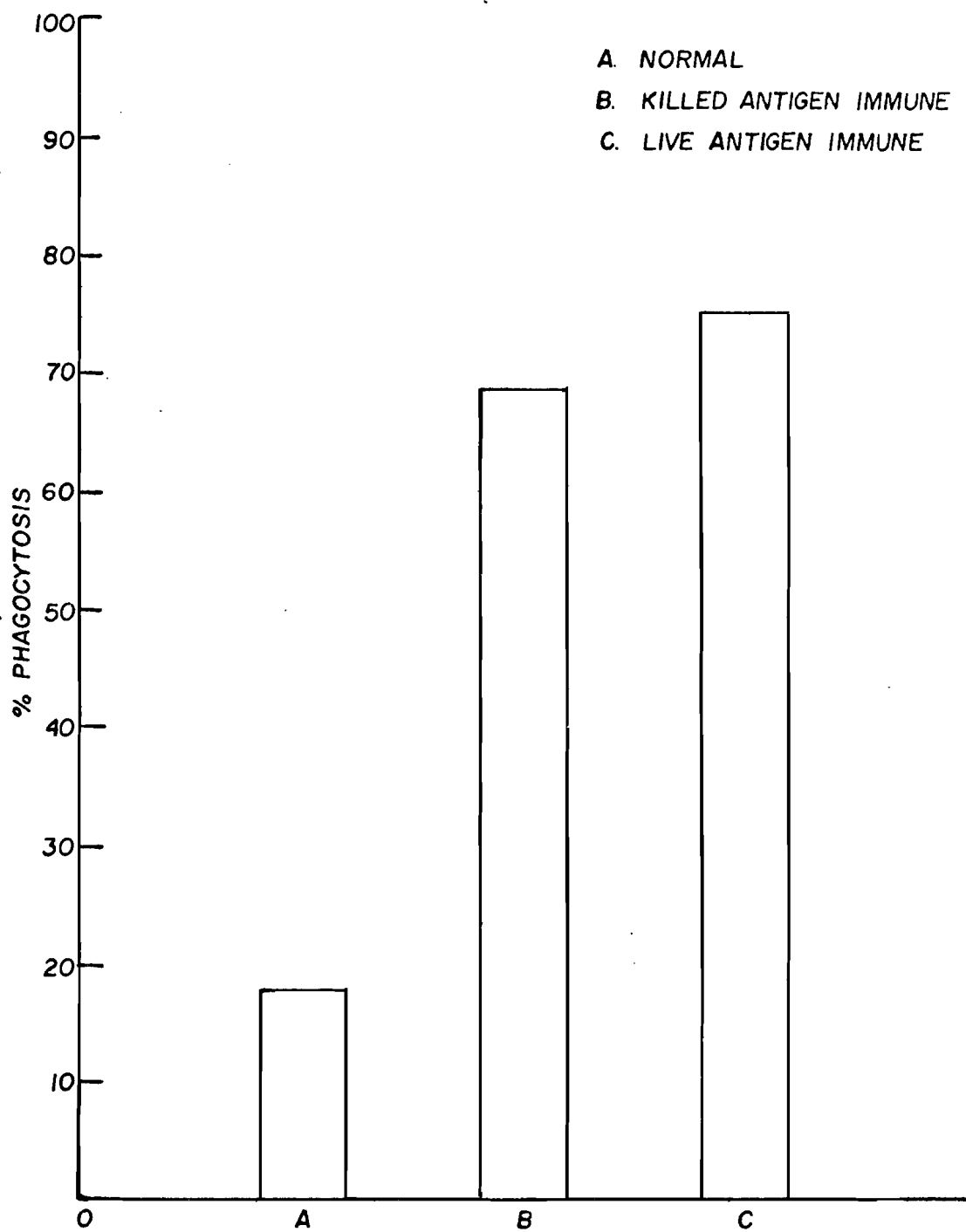
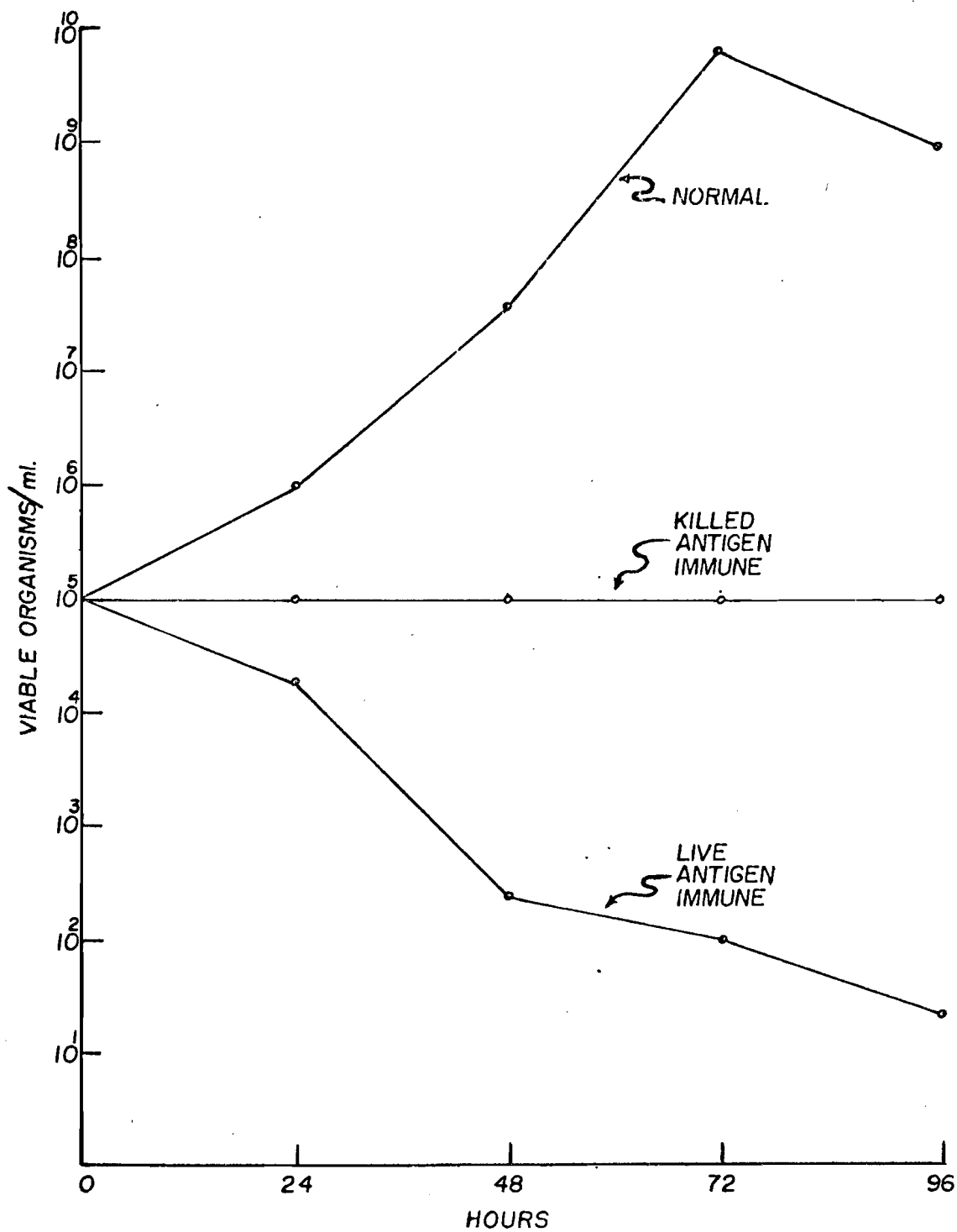


Figure 5: The multiplication of F. tularensis in mononuclear cell cultures prepared from rats immunized with live and dead F. tularensis.





The transfer phenomenon was found to occur in both immune cell systems. The phagocytic index of donors as compared to the recipients was so similar that the difference was insignificant (Figure 6). The viable count study demonstrated that the recipients were less effective than their donor counter-part, but even so, the live antigen immune lysate recipient cells were more effective in suppressing intracellular multiplication than were the killed antigen immune cells (Figure 7).

A study was done utilizing mouse cells, to check for the feasibility of the transfer phenomenon in another species. Previously it had been shown that one to two viable organism of F. tularensis, Schu strain, was fatal to mice, therefore only killed antigen immunization could be used for their immunization. The transfer phenomenon did occur as indicated by the increase in per cent phagocytosis as compared to the normal. Even though the increase was small as compared to rat cells, it was considered to be significant because it could be duplicated (Figure 8).

#### Species Specificity of the Immune Transfer Phenomenon

In his work, Stevenson (1967) obtained some indication that the transfer phenomenon might be species specific. He prepared a live antigen immune rat cell lysate, injected it intraperitoneally into a normal mouse, and after 13 days

Figure 6: Induction of opsonin activity in normal rats injected with cell lysates prepared from rats immunized with live and dead F. tularensis.

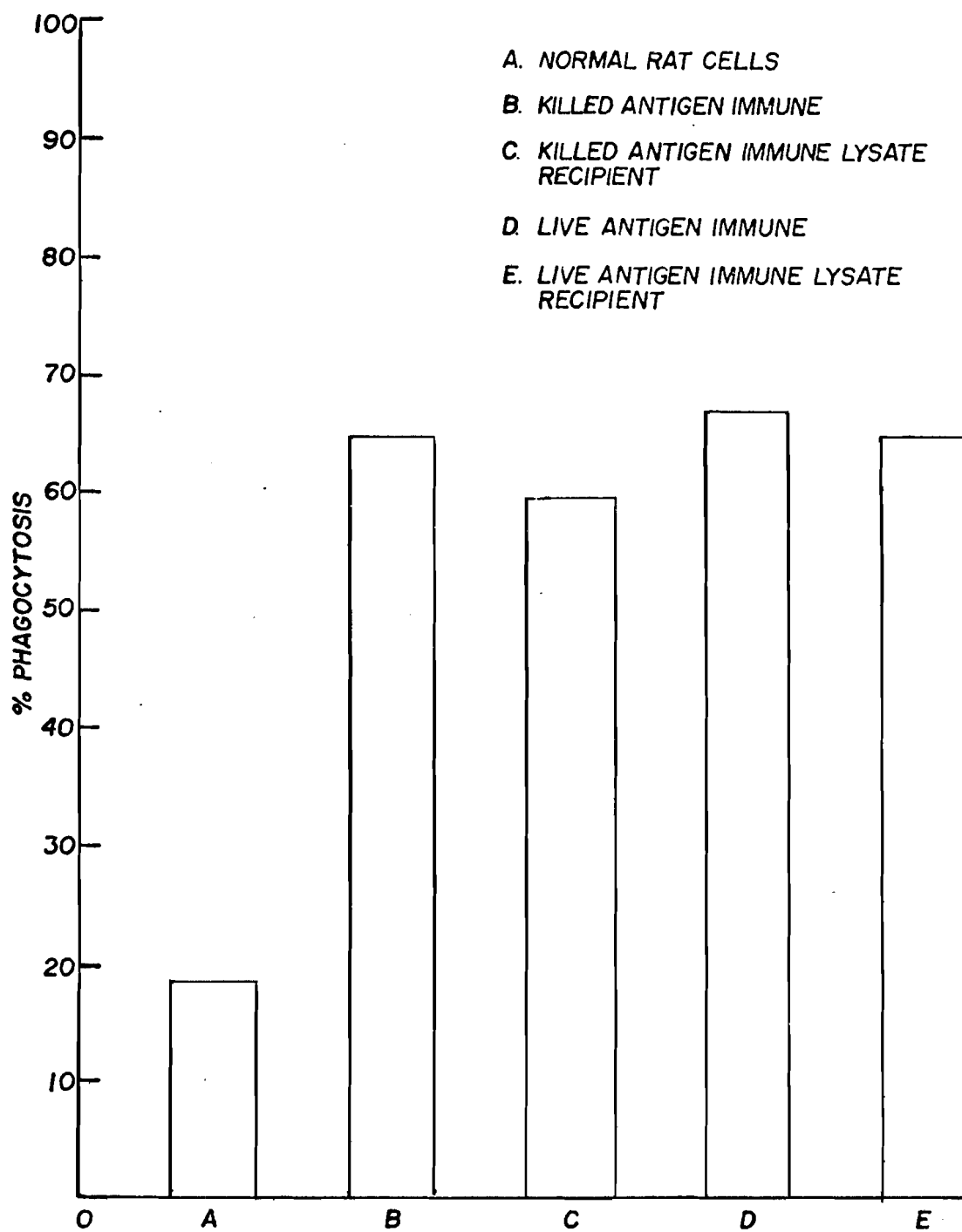


Figure 7: The multiplication of F. tularensis in mononuclear cell cultures prepared from recipients injected with cell lysates from rats immunized with live and dead F. tularensis.

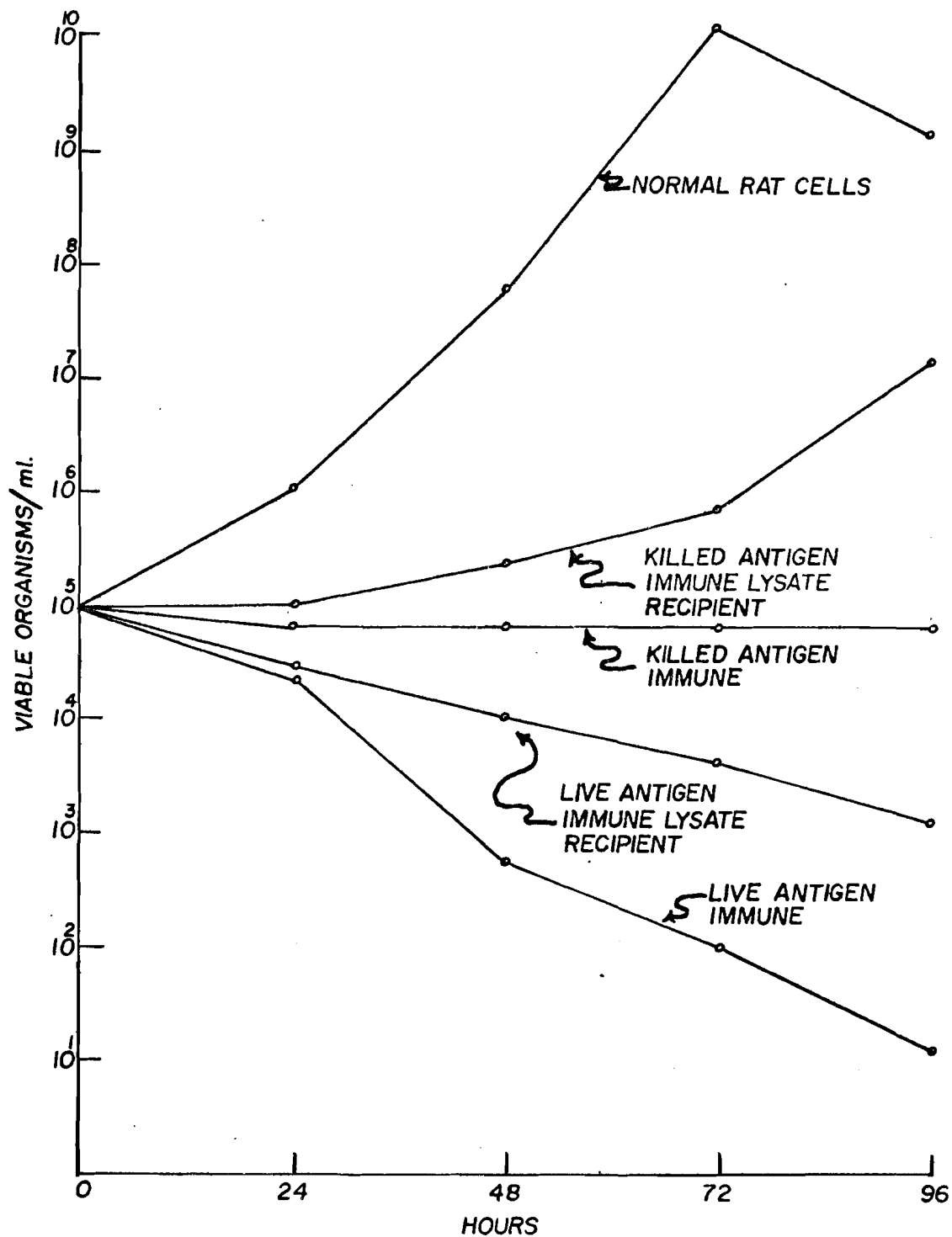
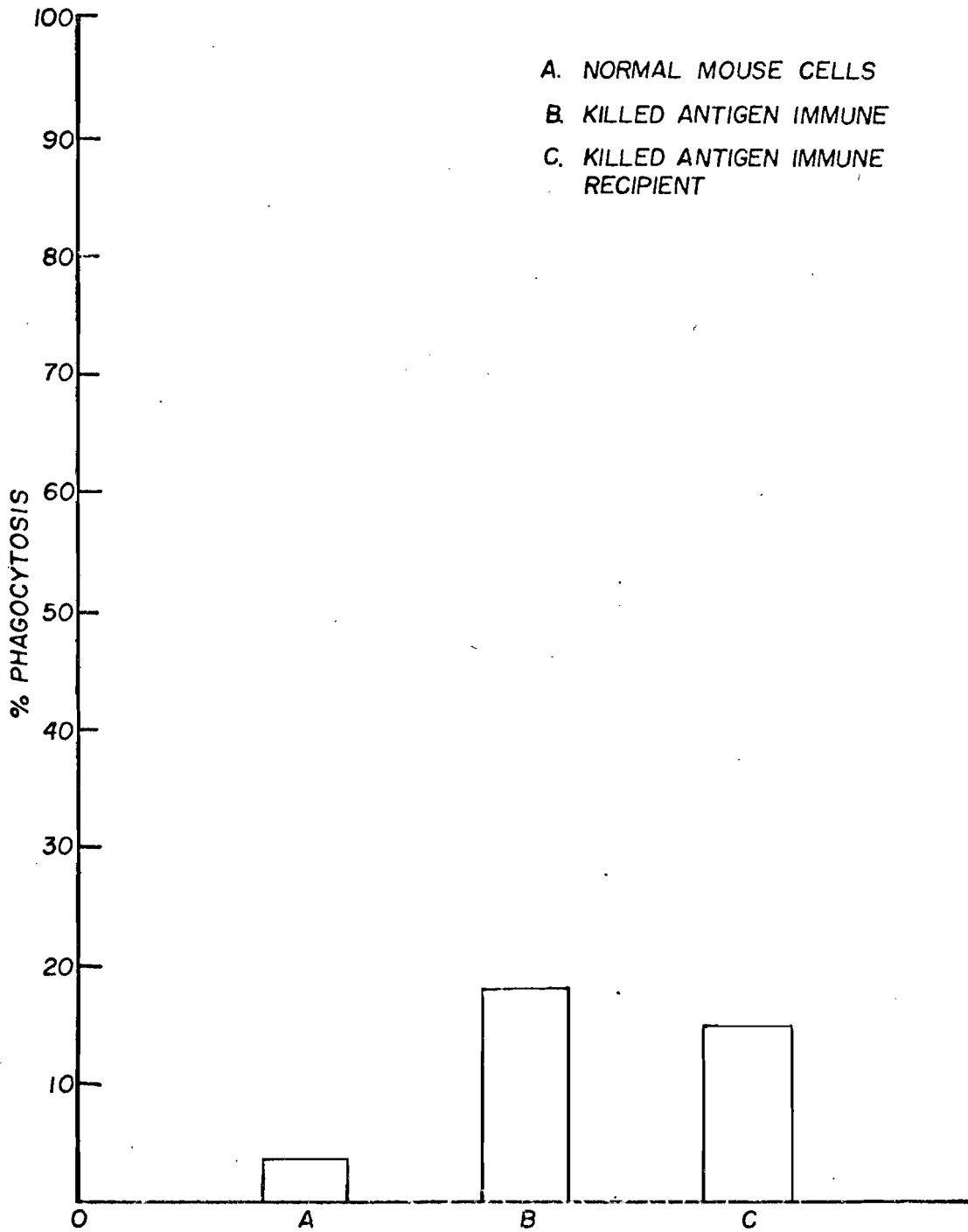


Figure 8: Induction of opsonin activity in normal mice injected with cell lysates prepared from mice immunized with dead F. tularensis.





received no significant increase in per cent phagocytosis of the recipient cells as compared to normal mouse cells. To confirm Stevenson's (1967) finding, killed and live antigen immune rat cell lysates were prepared and injected intraperitoneally into normal rats and mice. No activity was detected in the recipient mouse cells beyond that of normal mouse cells. The rat to rat transfer acted as a control, demonstrating the transfer factor to be present and active (Figure 9).

A killed antigen immune mouse cell lysate was prepared and injected into normal recipient mice and rats. This time an increase in activity was detected in the recipient mouse cells but not in the recipient rat cells, therefore confirming species specificity (Figure 10).

#### Centrifugal Fractionation of Immune Cell Lysate

A live antigen immune rat cell water lysate was prepared, fractionated by centrifugation, and the different fractions injected into normal rats. The fractions obtained were 2,000 xg supernatant (force for preparation of immune lysate), 15,000 xg pellet and supernatant, and 105,000 xg pellet and supernatant. The lysate was centrifuged at 2°C for 10, 20, and 30 minutes respectively. The lysate contained 0.24 M. sucrose and 0.001 M MgCl<sub>2</sub> to keep the microsomes intact.

Figure 9: Failure of rat immune cell lysate to induce opsonin activity in mice.

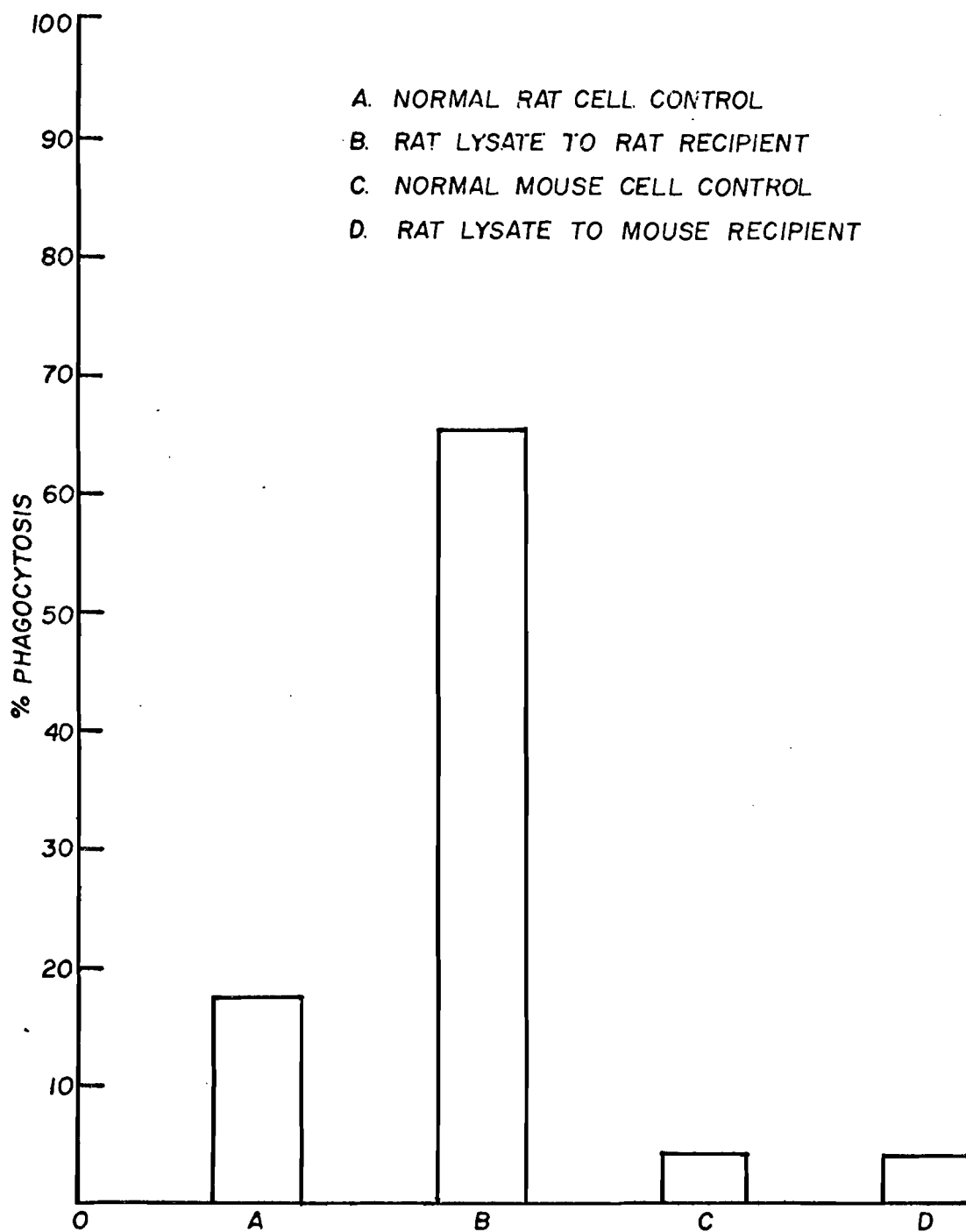
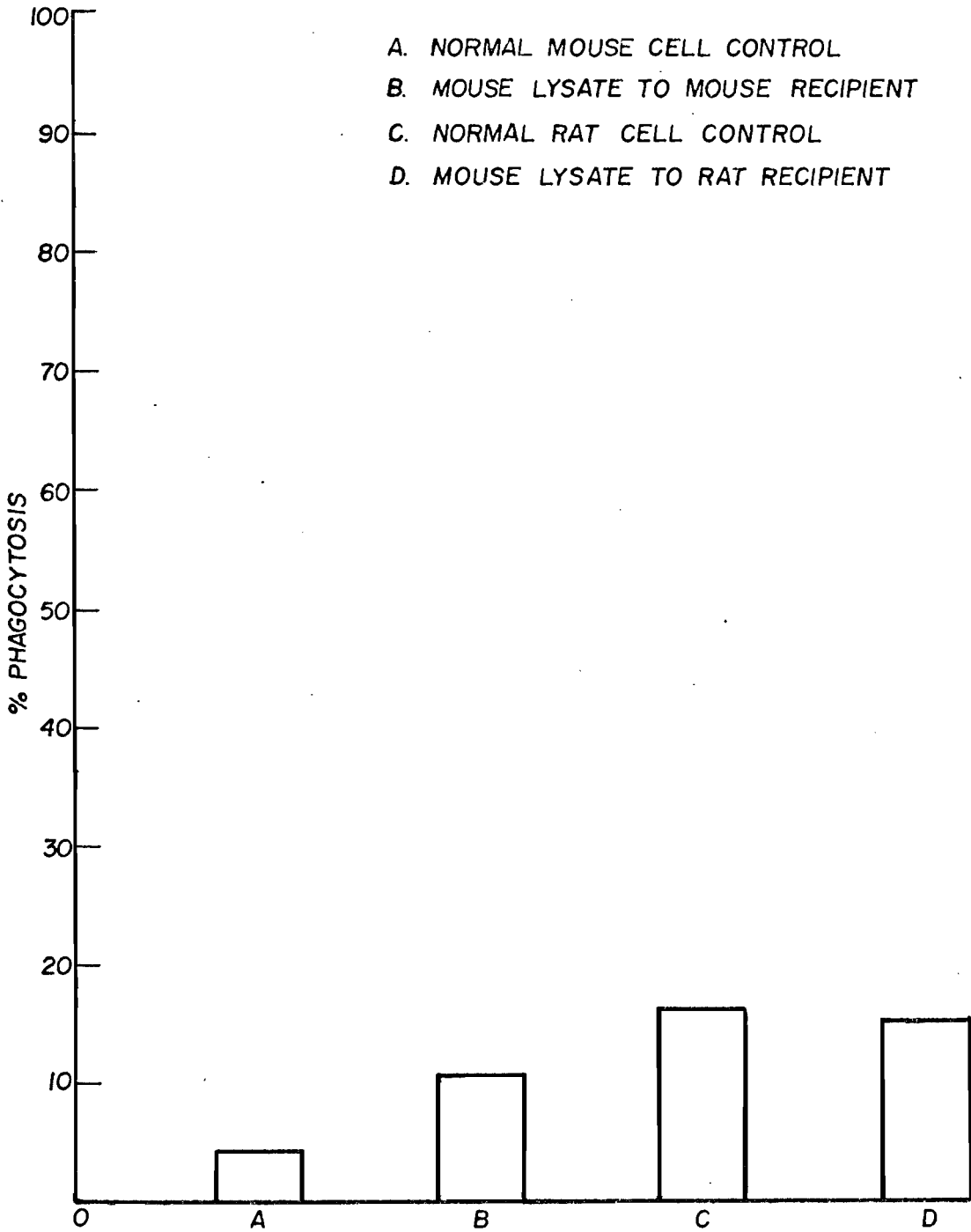


Figure 10: Failure of mouse immune cell lysate to induce opsonin activity in rats.



The pellets were resuspended in a quantity of distilled de-ionized water containing sucrose and  $MgCl_2$ , equal to that of the supernatant drawn off. The recipient normal rats received two ml intraperitoneal injections of a particular fraction.

The 2,000 xg supernatant contained organelles, nucleic acids, and some cellular debris. The 15,000 xg centrifugation resulted in sedimentation of remaining cellular debris and mitochondria, leaving microsomes, lysosomes, and nucleic acids still suspended. The remaining cell organelles were sedimented at 105,000 xg with the nucleic acids still suspended (Packer, 1967).

Phagocytic studies of cells obtained from the recipients of the various fractions demonstrated activity being transferred only by the supernatants of the 2,000 xg and 15,000 xg fractions. The other three fractions demonstrated no phagocytic activity greater than that found with normal rat cells (Figure 11).

The viable count study (Figure 12) demonstrated the recipient cells of the normal immune lysate as the only system suppressing intracellular multiplication. The ability of the 15,000 xg supernatant to suppress intracellular multiplication is yet to be determined. Technical difficulties have prevented an accurate assay after 36 hours. The recipients of the other three fractions showed no significant

Figure 11: A study of the induction of opsonin activity in recipients injected with different centrifugal fractions of immune cell lysate.

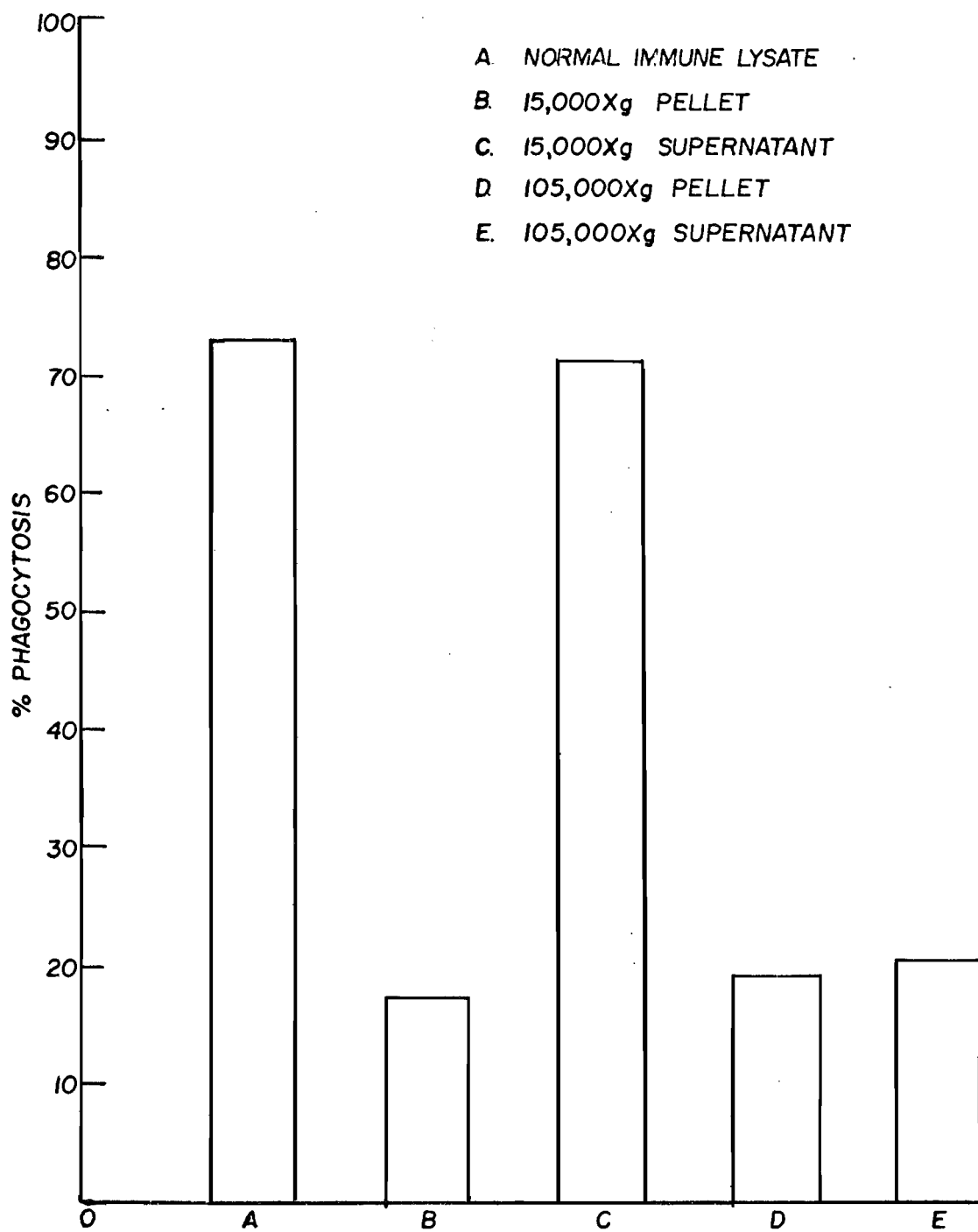
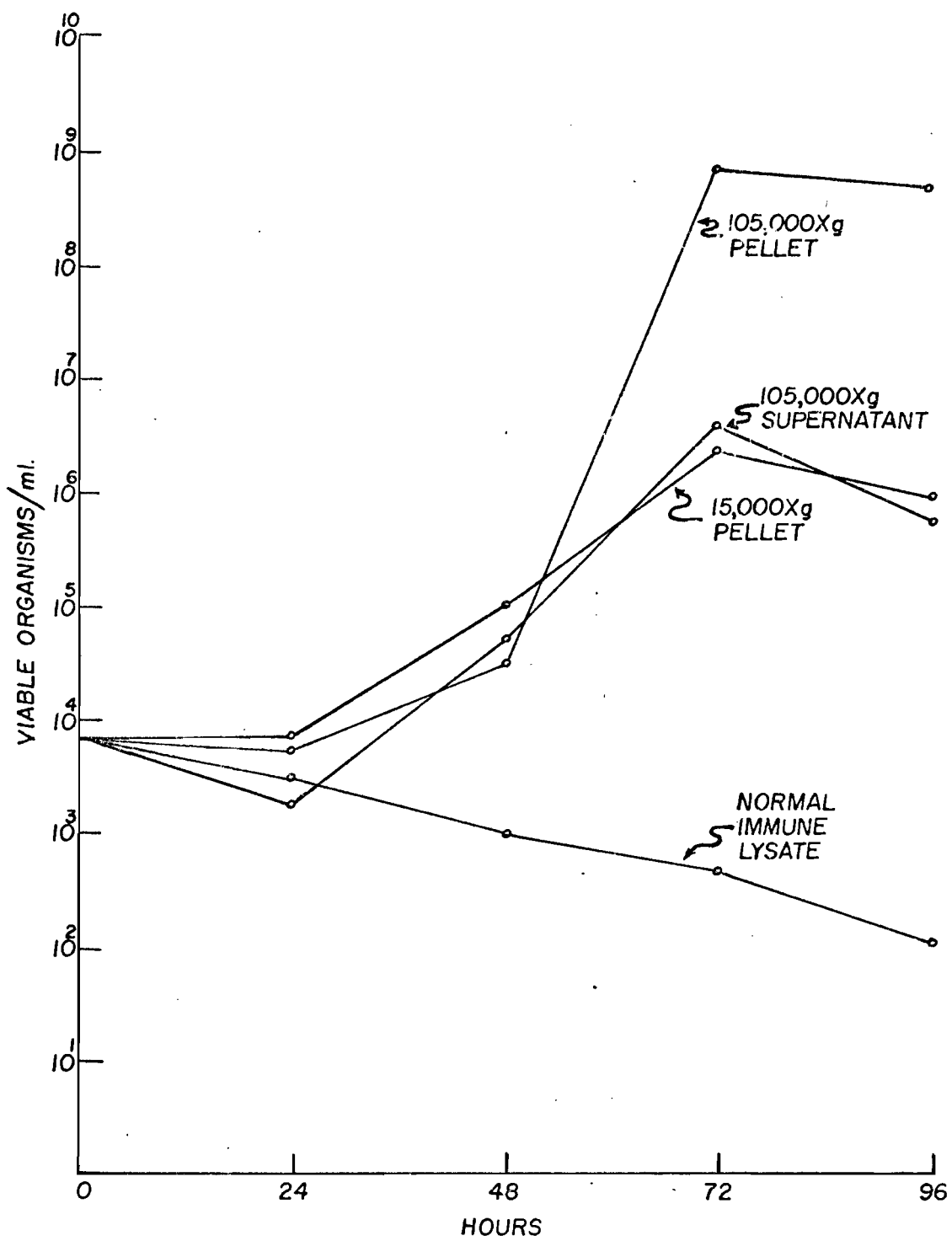




Figure 12: The multiplication of F. tularensis in mononuclear cell cultures prepared from recipients injected with different centrifugal fractions of immune cell lysate.



suppression of intracellular multiplication, for after 72 hours almost total cellular destruction had occurred.

### In Vitro Induction of Cellular Protection

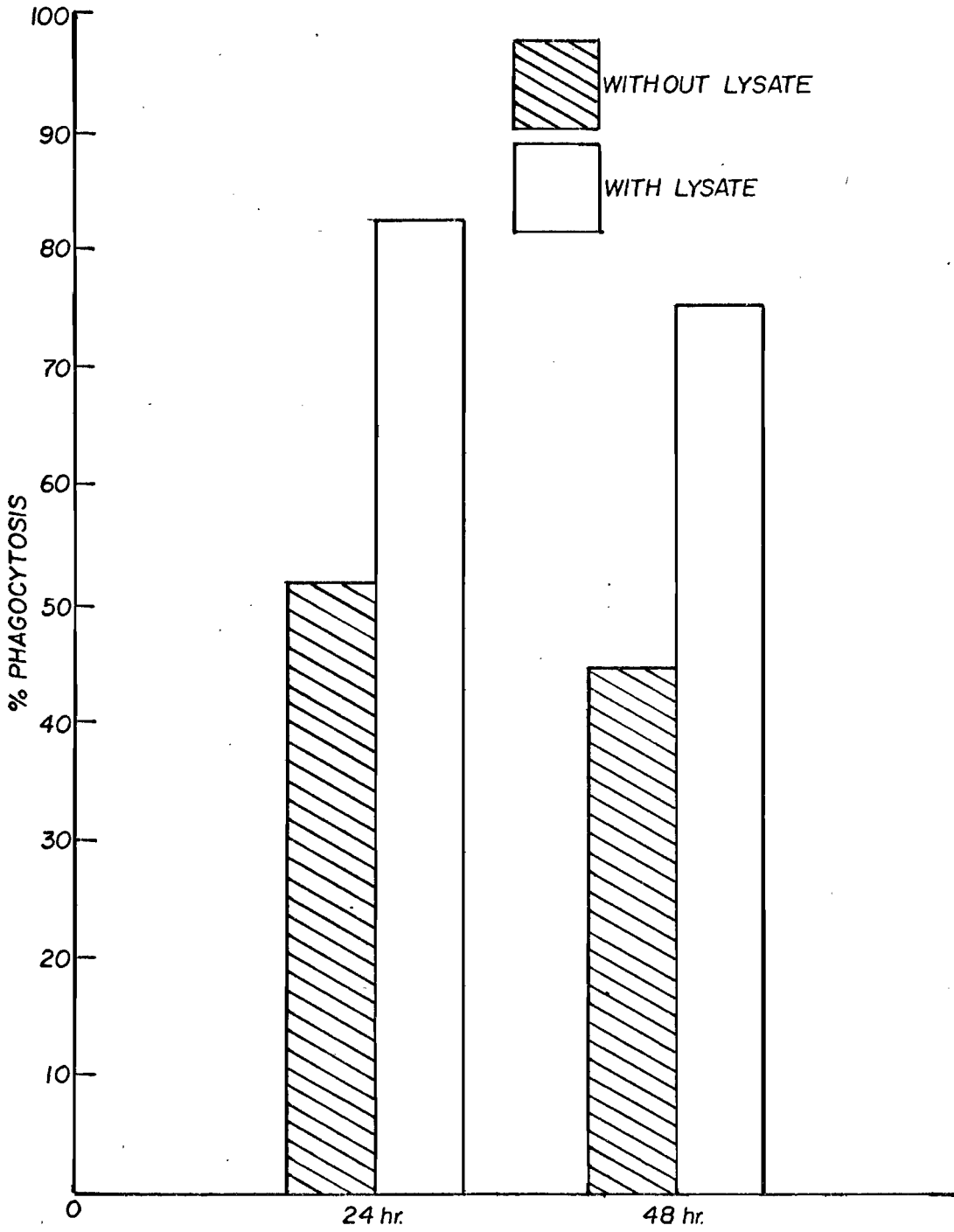
Workers (Fishman, 1959; Mitsuhashi and Saito, 1962) have successfully induced cellular protection in vitro by stimulating primary responses or transferring the protection by passive transfer. An attempt was made to stimulate cellular protection in vitro by using an immune cell lysate in the cell culture medium.

An immune cell lysate was prepared and normal rat cells were harvested. The normal cells were planted and allowed to settle. The medium in the coverslip tubes was replaced by Scherer's diluted to a 1X solution by either immune lysate or water. The replacing medium also contained 30% heated calf serum. At 24 and 48 hours two representative coverslip tubes from each of the two systems was infected with F. tularensis, at a 1:5 ratio, and a phagocytic study performed.

The results showed (Figure 13) a significant difference between those cells incubated in Scherer's plus immune lysate and the ones incubated in Scherer's plus water. At both time periods the lysate treated cells showed significantly more phagocytic activity than the controls.

Further studies of the response included the addition

Figure 13: Enhancement of the phagocytosis of F. tularensis by normal rat cells cultured in Scherer's medium supplemented with normal calf serum and immune cell lysate.



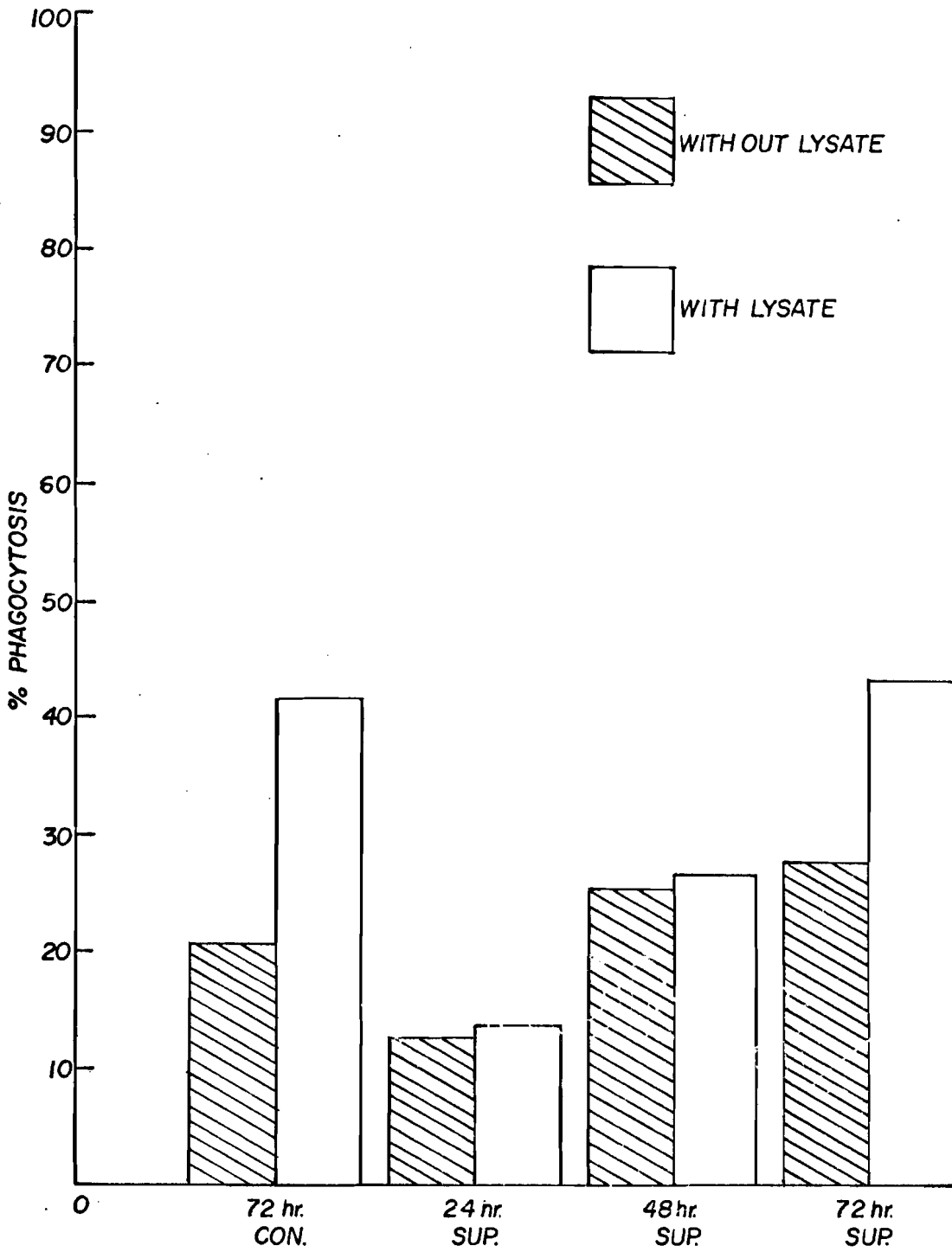
of 0.1 ug hydrocortisone/ml (Stecher and Thorbecke, 1967) with the deletion of heated calf serum in the systems. Systems were set up as above except that the incubating medium was changed every 24 hours through a 72 hour period with the phagocytic activity of the medium assayed daily on freshly harvested normal rat cells. The normal rat cells on which the medium was incubated through the 72 hours were also quantitated for their phagocytic activity at the termination of the experiment.

The phagocytic study (Figure 14) did not demonstrate a significant difference between the experimental and control systems before 72 hours. The cells hosting the medium produced a phagocytic index at 72 hours, which was similar to the phagocytic index of the medium removed at that time period. The data demonstrates that the immune cell lysate was capable of stimulating some cellular protection.

#### Effectiveness of Lysates Prepared by Different Methods

Peritoneal macrophages were harvested from immune rats, washed and adjusted to  $10^7$  cells per ml in distilled de-ionized water. The cell suspension was divided into three portions with each suspension being lysed by a different method. The methods used were water, freeze-thaw, and sonication. The lysates were injected into normal rats and the cells harvested after 13 days to quantitate the

Figure 14: A study of the phagocytosis of F. tularensis by freshly harvested normal cells bathed in supernatants of cells cultured in the absence and presence of immune cell lysate.





immunogenic activity.

The phagocytic activity was found to be the greatest in the water lysate recipients with the freeze-thaw lysate recipients only slightly less efficient. The sonicated preparation stimulated about one-half the activity stimulated by the water lysate, but it was still greater than normal rat cells (Figure 15).

The freeze-thaw and water lysate recipients demonstrated equal ability in suppressing intracellular multiplication through 72 hours. After the 72 hour time period the results obtained from the freeze-thaw recipient were regarded as questionable. The sonicated lysate recipient was somewhat less efficient in suppressing intracellular multiplication than the other two systems (Figure 16).

#### Serial Transfer on the Immune Transfer Phenomenon

An immune lysate was prepared and injected intraperitoneally into normal rats. After 13 days the immune lysate recipient rats were sacrificed and their cells harvested. The harvested immune cells were divided into two portions, one to be made into a second lysate, and the other portion was quantitated for its immunogenic activity. The lysate was injected into a second recipient, the cells harvested after 13 days, and the procedure of transfer and quantitation repeated.

Figure 15: A study of the induction of opsonin activity in recipients injected with different immune cell lysate preparations.

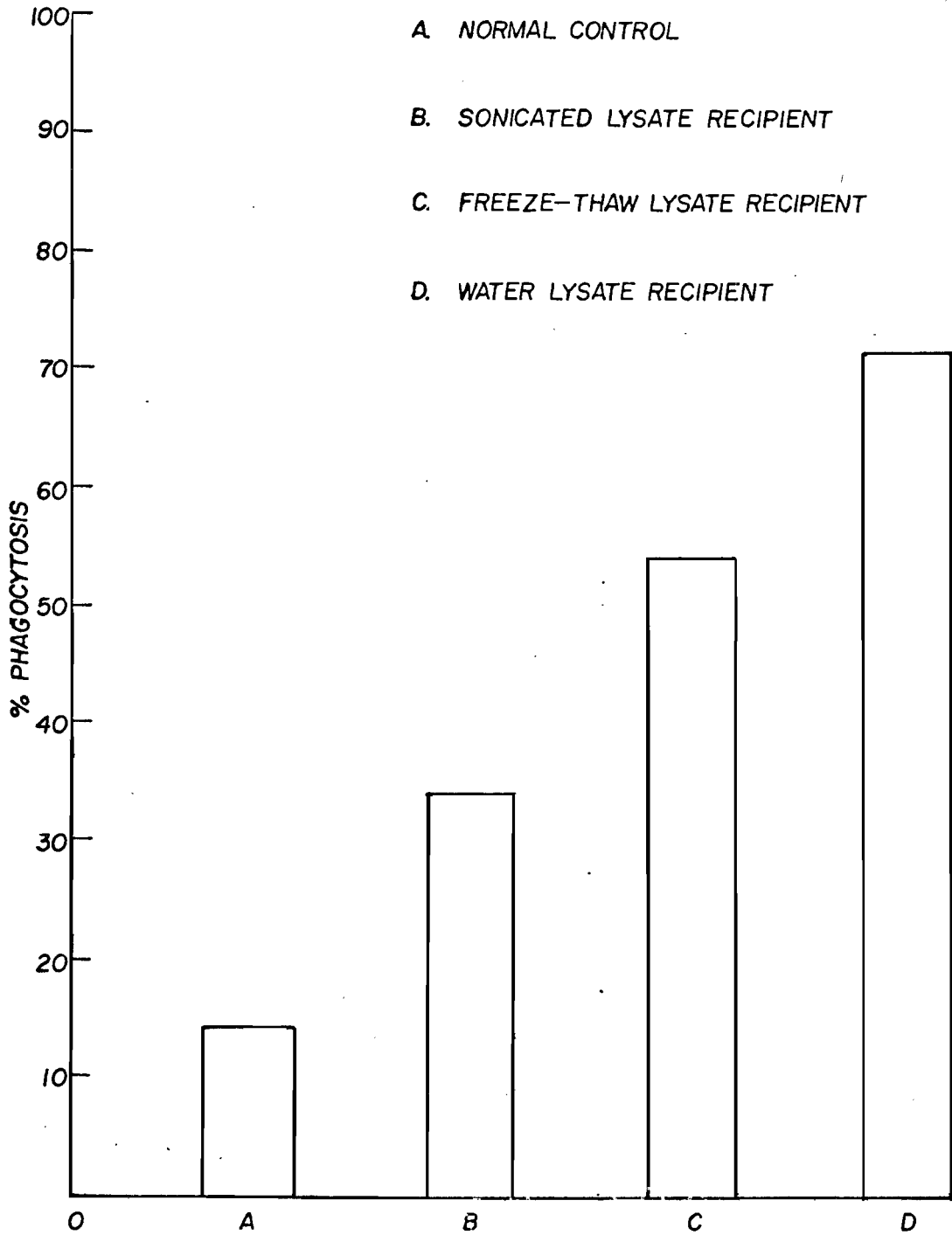
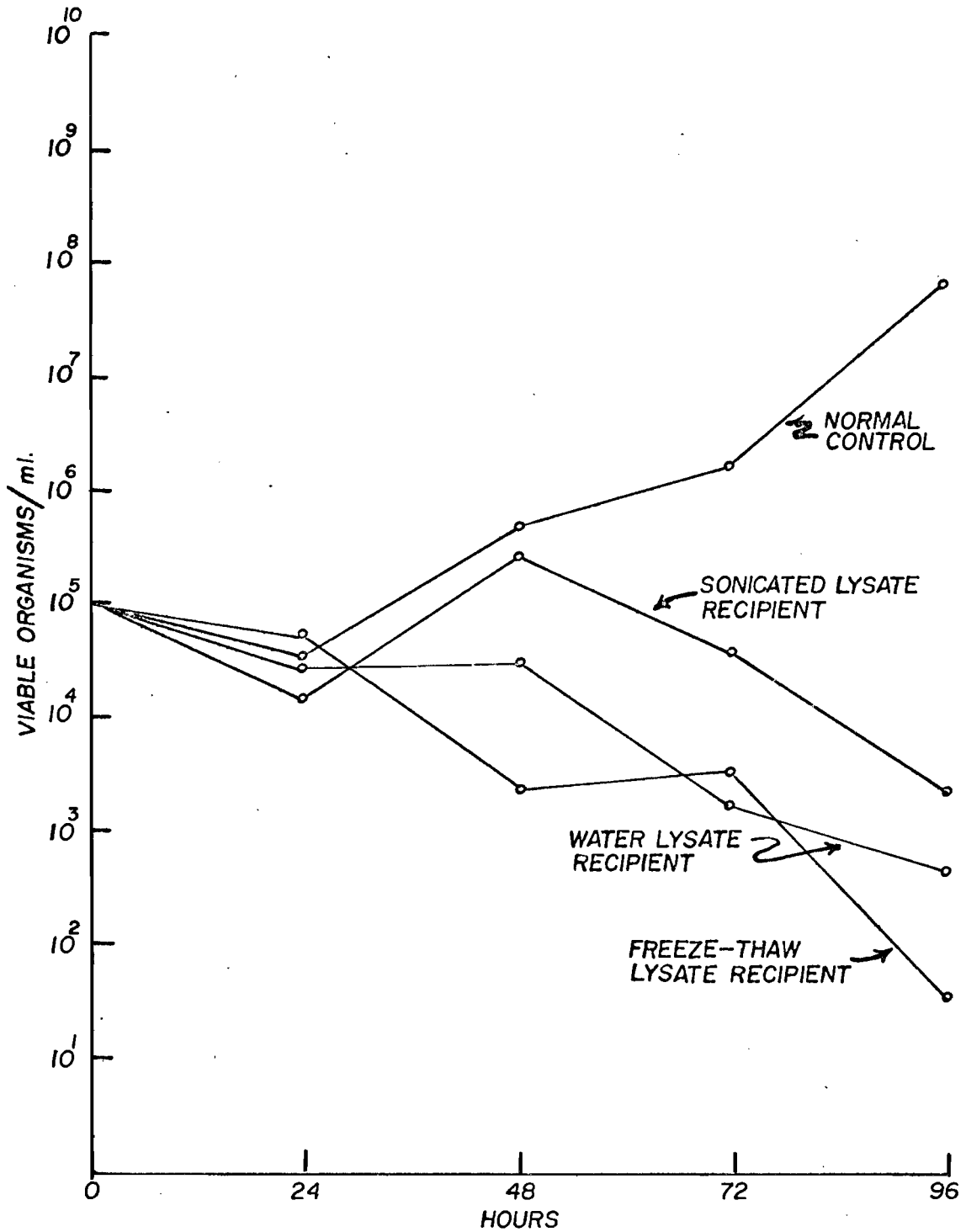


Figure 16: The multiplication of F. tularensis in mononuclear cell cultures prepared from recipients injected with different immune cell lysate preparations.



The first recipient demonstrated the phagocytic activity and ability to suppress intracellular multiplication as was expected. The second recipient showed no immunogenic activity beyond that of normal rat cells (Figures 17 & 18). It was therefore concluded that the transfer phenomenon was not possible with this system.

Figure 17: Failure to induce opsonic activity by the serial passage of immune cell lysates.

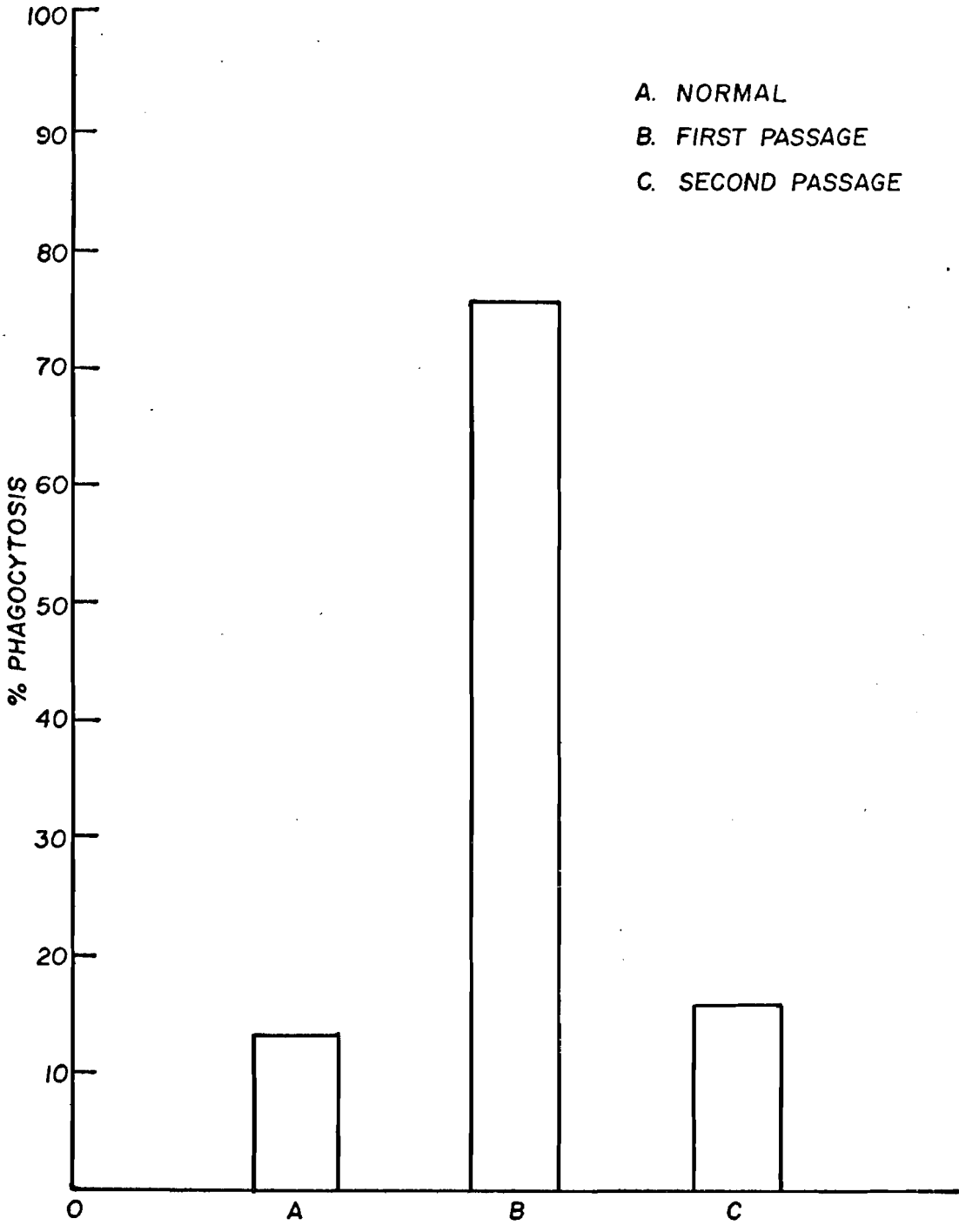
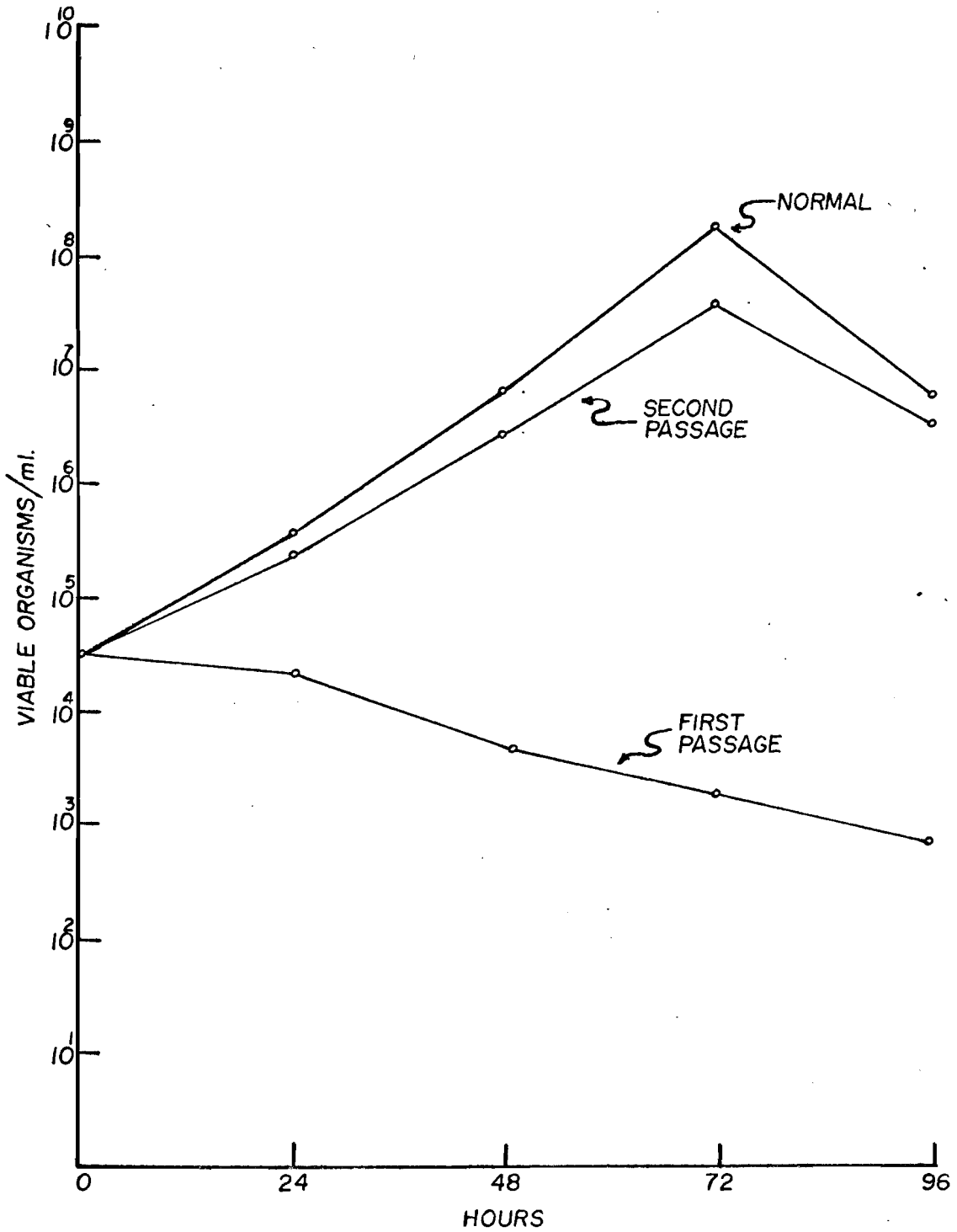




Figure 18: The multiplication of F. tularensis in mononuclear cell cultures prepared from first and second passage recipients of immune cell lysate.



## DISCUSSION

Recent results, some of which are reported in this paper, indicate that cells typical of the reticulo-endothelial system play a key role in immunological response. Although precise mechanisms have not been clarified, macrophages appear to respond to an antigenic stimulus by developing cellular immunity and "processing" the antigen (Uhr and Weissman, 1965; Nossal, 1966) followed by the transfer of immunogenic information to immunocompetent cells with subsequent initiation of antibody synthesis (Fishman, 1963).

In most instances immunological immaturity in the young or immunological deficiency in adult animals is attributed to the absence of immunocompetent cells. However, it was reported by Gallily and Feldman (1967) that immunological deficiency appearing after low doses of x-irradiation could be overcome by the administration of macrophages. They presented evidence that the immunological insult in adult mice produced by low doses of x-irradiation is not directed against the immunocompetent cell but is directed against the antigen-recognizing capability of antigen-processing cells, namely the macrophages. Argyris (1968) working with immunological immaturity in new born mice obtained comparable results.

The uptake of an antigen by peritoneal macrophages is followed by antigen degradation within two to five hours. Only 10% of the original content of the antigen remains in the immunogenic system, the remaining 90% is discarded (Unanue, 1968). The immunogenic structure of the remaining 10% is presently an open question in regard to whether it is complexed with an RNA or simply stimulates the synthesis of an informational RNA.

Fishman (1963) has demonstrated that antibody against T<sup>2</sup> bacteriophage is produced by lymph node cells upon addition of RNA extracted from phage-stimulated macrophage cells. Subsequent studies have revealed that the RNA derived from the macrophages contained antigenic fragments complexed with it (Friedman, 1965), and that this complex was immunogenic (Askonas and Rhodes, 1965). It is unsettled as to whether the production of specific antibody by lymphoid cells should be attributed to a messenger RNA derived from the macrophage RNA merely acts as an adjuvant to promote the immunogenicity of bound antigen (Friedman, 1965).

There is also incomplete agreement as to whether the macrophage cell manufactures a new kind of RNA following antigen phagocytosis, or utilized an RNA already present (Halac, 1964). Raska and Cohen (1967) demonstrated an acceleration of labelled uridine incorporation into RNA of mouse peritoneal cells during incubation with sheep red

blood cells in vitro. The labelled RNA forms a specific molecular hybrid with mouse DNA in a manner that suggests the synthesis of a new species of macrophage RNA upon the addition of antigen. Halac (1964) reported a change in the base composition of total RNA from rat peritoneal macrophages following the phagocytosis of bovine serum albumin. It appeared that the altered base ratios were attributed to the synthesis of a new species of RNA, but no effort was made to determine which species of RNA was responsible. Fishman and Adler (1967) obtained similar results, indicating macrophages, possibly of a select type, produced a specific RNA in response to antigen stimulation. Such RNA, free of detectable antigen, evoked antibody formation in lymph node cells.

Gottlieb and Glisin (1967) suggested that "immunogenic RNA" extracted from macrophages is not a unique species of RNA. Through experiments with RNA extracted from macrophages following the phagocytosis of different bacteriophage antigens they indicated that the binding sites of the active RNA to macrophage DNA were not unique for a given antigen. In contrast Raska and Cohen (1968) reported an antigen-specific RNA synthesis. However, they were working with lymphocytes incubated in vitro with foreign red blood cells rather than macrophages.

Monocytes have been reported to exhibit cellular immunity in infections with intracellular organisms including

F. tularensis. Thorpe and Marcus (1964a) obtained evidence that peritoneal macrophages from rabbits immunized with viable F. tularensis were more effective in the ingestion and intracellular destruction of the organism in an in vitro system than were similar cells from normal rabbits. Specific extracellular antibody was shown to play only a minor role in the in vitro resistance demonstrated by these workers. Thorpe and Marcus (1964b) further reported that peritoneal cells from immunized mice and guinea pigs exhibited resistance to the organism similar to that exhibited by immune rabbit macrophages. It was concluded that cellular factors were of primary importance and that humoral factors were of secondary importance in immunity to F. tularensis.

The mechanism responsible for the ability of the immune phagocytes to inhibit the intracellular growth of the organism remains unknown. A step forward in understanding the mechanism involved was contributed by Fong et. al. (1963) when they demonstrated that cellular resistance to Mycobacterium tuberculosis could be passively transferred to normal rabbits by a ribosomal fraction prepared from macrophages of immune rabbits. Because their studies produced no evidence of antigenic stimulation and because they could serially transfer cellular resistance through several animals, a procedure which would dilute a non-replicating antigen, they suggested that a replicating form of RNA from immune cells

was responsible for the transfer phenomenon. Subsequent in vitro experimentation by Saito and Mitsuhashi (1965) also demonstrated the transfer factor to be associated with the ribosomal fraction of immune cells.

The fact that both cellular immunity and induction of antibody have been established as an RNA dependent phenomenon suggest the two responses may be inter-related. Japanese investigators (Kurashige, 1967; Mitsuhashi, 1967) produced work that strongly supports this idea by the demonstration that immune cells resisted cell degeneration and suppressed the intracellular multiplication of the organism Salmonella enteritidis in the absence of antibody. They described a cellular associated antibody that, when extracted from the cells, had specific inhibitory activity in the presence of complement and lysozyme. On the basis of their results, they suggested that mononuclear phagocytes were capable of antibody production.

Dead and live antigen-immunized rat peritoneal cells demonstrated cellular immunity plus specific opsonins. Fractions from live or dead antigen-immunized monocytes were capable of transferring cellular protection to recipients. The immunized recipient cells were not as effective as suppressing intracellular multiplication as their respective donors, but their phagocytic indices were very similar to the donors. The live antigen-immune cell system and its

recipients were the most effective forms of immunity. The live antigen immunization resulted in effective protection whereas the dead antigen immunization was only capable of prolonging the cells fatal termination.

Using water lysates of immune cells from rat donors, an RNAase sensitive fraction was shown to be capable of inducing both cellular resistance and specific antibody in normal rat recipients. The ability of the immune cell lysate to transfer cellular protection was destroyed by treating it with a minimum concentration of 0.1 ug of RNAase/ml. These findings differ from those reported by Fong (1962, 1963) in several respects. In contrast to the findings reported here, Fong (1963) failed to find specific antibody in the immune lysate recipients. Furthermore, our attempts to serially transfer the immune phenomenon failed, an important fact relating to the interpretation of the mechanism involved.

As previously stated, it is unsettled as to whether the production of specific antibody by lymphoid cells should be attributed to a messenger RNA derived from the macrophage cell (Adler, 1966; Fishman and Adler, 1967) or whether the macrophage RNA merely acts as an adjuvant to promote the immunogenicity of bound antigen (Friedman, 1965). Results presented in this paper demonstrated that the transfer of immune cell fractions between homologous species induced



cellular immunity and antibody production, but attempts to transfer immunity in a like manner between heterologous species was unsuccessful. Therefore, it was concluded, at least for rats and mice, that the transfer of the immune phenomenon was species specific. Fishman (1963) while working with induction of antibody in vitro also described his inducing factor as species specific. These results clearly indicate that the role of the RNA in the immune phenomenon is more than a simple adjuvant. Fong (1963) was able to demonstrate the transfer of cellular protection with heterologous species. The transferring material was described as free of viable bacilli to which the histiocytes were immune. The difference in the results may be due to differences in the systems employed in the two studies.

For additional insight into the mechanism of the transfer phenomenon an experiment for in vitro induction of cellular protection was designed. Since the early days of immunology many workers have tried to initiate and maintain in vitro an immunological response leading to the formation of antibody by cells from nonimmune animals. Various laboratories have succeeded in maintaining tissues and cells actively producing antibody in culture for various lengths of time (Hannoun, 1966), while others have been able to bring about antibody production in quiescent tissue and cells previously stimulated in vivo by in vitro contact with

specific antigens (Michaelides, 1963; Harris, 1965). Another line of investigation was to stimulate un-committed cells in vitro before transferring them to incompetent recipients whose presumed role would be to sustain the life of the "grafted" antibody-producing cells (Harris, 1965). A closer approach to the complete in vitro conditions was achieved by using peritoneal chambers in recipients that played the role of "living incubators" for the immunocytes (Capalbo, 1964; Bussard, 1966). Some authors have been able to initiate a complete in vitro stimulation of tissue fragments (Globerson, 1966; Tao, 1966; Saunders, 1966), or of isolated cells from nonimmune animals (Fishman, 1961; Friedman, 1965). In the latter case the antibody production was demonstrated either by detection of antibody activity in the culture fluid or associated with the cells.

Results presented in this paper demonstrated the in vitro induction of opsonin activity, stimulated by incubating normal rat cells in the presence of immune rat cell lysate. Hydrocortisone was used in the media as a serum substitute. Stecher (1967) described hydrocortisone as an effective substitute for serum in the case of rat cells but not mouse cells. The control consisted of water diluted Scherer's whereas the experimental system consisted of immune cell lysate diluted Scherer's. A significant difference in opsonin activity was observed between the control and

experimental systems after 72 hours. It was also after the time interval in which maximum opsonin activity was observed. The induction of opsonin activity cannot be attributed to viable organisms, for the lysate was tested and found to be sterile.

Further studies designed to further explore the nature of the immune lysate found that the preparation and the centrifugal fraction injected were two very important criteria for effective transfer of the immune phenomenon. Recipients of a freeze-thaw prepared lysate were more effective in suppressing intracellular multiplication than recipients of the water-prepared immune lysate. The reverse was true, however, with regard to phagocytic activity in which the recipients of water-prepared immune lysate manifested a higher phagocytic index than those receiving a freeze-thaw prepared lysate. The sonicated preparation recipients were the least effective of the three in inducing cellular protection.

Different methods of lysate preparation induced varying degrees of cellular protection. This correlation has been suggested as having to do with the breakdown of the cellular membranes, such as those that make up the lysosomes which contain RNAase plus other hydrolytic enzymes. Sonication was the most effective method for cellular destruction, followed by freeze-thaw with the least

destructive being water lysis. The effectiveness of the method used is inversely proportional to the amount of cellular protection induced.

Further investigation of the immune lysate was pursued through centrifugal fractionation. The centrifugal fractionation performed on the immune cell lysate demonstrated no activity induced by fractions containing only nucleic acids or organelles. The 15,000 xg supernatant which contained both elements demonstrated activity equal to that of normal preparation of immune lysate recipients. If the results could have been anticipated the 105,000 xg supernatant and pellet should have been pooled to determine whether a complementary factor had been divided or if the activity had been destroyed by a physico-chemical reaction in the process of centrifugation.

## SUMMARY

Water lysates prepared from peritoneal cells taken from immune animals will induce both cellular resistance and opsonin formation when injected into normal rat recipients. Although lysates prepared from animals immunized with dead vaccines induce comparable opsonin responses, they fail to transfer cellular protection equivalent to that obtained with cell lysates from animals immunized with live organisms.

The method by which an immune lysate was prepared and the centrifugal fraction injected constitutes two very important criteria in the successful transfer of cellular protection.

The treatment of immune cell lysates with RNAase prior to injection destroys the activity of the lysate indicating that it is an RNA dependent phenomenon.

Failure to obtain serial transfer of activity in our experiment indicated that it is not a self-replicating factor as reported by Fong (1962).

Although the question remains open in regard to the nature of the active factor present in immune cells, that is to say, whether it is RNA or an RNA-antigen complex, failure to transfer activity between species suggests that the role of RNA may be more critical than a simple adjuvant function.

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