PASSIVE TRANSFER OF IMMUNE MECHANISMS WITH RIBONUCLEASE-SENSITIVE FRACTIONS OF IMMUNE CELL LYSATES

A Thesis

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by

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J. R. S.

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INTRODUCTION

Ribonucleic acid (RNA) extracts of certain cell types have recently been implicated in immunological phenomena. These ribonuclease-sensitive extracts have been shown to be involved in the *in vitro* induction of antibody formation, in the passive transfer of transplantation immunity and in the passive transfer of cellular immunity.

Early attempts to induce a primary antibody response *in vitro* failed uniformly (Stavitsky, 1961). In these studies, the antigen was merely introduced into the culture medium in which the lymphoid cells were bathed. In 1959, Fishman, using a somewhat more complicated system, was first successful in the induction of a primary antibody response *in vitro*. He incubated the test antigen, bacteriophage T2, with normal rat macrophages for 30 minutes at 37 C, washed the macrophages and homogenized them. Five days after the filtrate of this homogenate had been added to normal rat lymph node cells in tissue culture, neutralizing activity specific for T2 was detected in the culture medium. The titer of this neutralizing activity increased until day 11, when the cells apparently began to lose viability. Optimum results were obtained with a T2-macrophage ratio of 1 to 1000. Control systems, to which untreated T2 or homogenates of untreated macrophages were added, showed no neutralizing activity. The induction appeared to be somewhat species-specific, since pretreatment of the phage with rabbit macrophages or with HeLa cells could not replace pretreatment with rat macrophages when the homogenates were added to rat lymph node cells. These results indicated that interaction between

macrophage and antigen was an essential step in the *in vitro* induction of antibody synthesis.

In a subsequent study, Fishman (1961) showed that the neutralizing activity was due to antibody, since the material responsible for the activity was precipitated at the isoelectric point of serum globulin and since rabbit antibody against rat globulin inhibited the activity. Strictly *in vitro* work gave positive results only 20% of the time; therefore experiments, in which lymph node cells treated with T2-macrophage mixture were injected into chick embryos, were included in this study. When incubation of the T2-macrophage mixture was terminated at 10 minutes, the resultant homogenate was inactive. However, if the macrophages were incubated alone for an additional 20 minutes after this 10 minute period with antigen, an active homogenate was obtained. Thus, the macrophage was further shown to act upon the antigen in some way. The homogenate could be inactivated by heating at 80 to 100 C for 15 minutes, as well as by the addition of ribonuclease or streptomycin. Fishman (1961) postulated that some form of RNA comprised the active portion of the homogenate.

In a subsequent study, Fishman, Hammerstrom and Bond (1963) used radioautography to show that low molecular weight macrophage RNA labelled with tritiated cytidine was, indeed, incorporated into lymph node cells in culture. They used RNA from untreated macrophages only, so it was not possible to know whether the RNA fractions incorporated were the same as those involved in the *in vitro* induction of antibody formation.

In further studies (Fishman and Adler, 1963), x-irradiated non-I rats which received diffusion chambers charged with normal lymph node cells and T2-macrophage homogenate showed circulating antibody specific for T2. Chambers charged with homogenate alone led to circulating antibody specific for T2 only in non-irradiated recipients. Neither group of rats gave a titer when homogenates of untreated macrophages were used in the chambers. When phenol was used to extract the RNA from the homogenates, all of the activity of the homogenates was contained in the low molecular weight RNA fraction. This extract could be inactivated by ribonuclease; hence, two probable mechanisms were postulated. (1) The RNA might induce antibody synthesis by virtue of mRNA activity within the recipient lymph node cells—"transformation". (2) The RNA extract might contain bound antigen that is responsible for the induction process.

The results of Fishman (1961) and Fishman and Adler (1963) were confirmed by Friedman, Stavitsky and Solomon (1965). In addition, they found that when the T2-macrophage mixture was incubated at 0 or 56 C, the resultant RNA extracts were inactive. Thus, the active role of the macrophage in this system was more clearly established. Pretreatment of the extract with ribonuclease did not completely abolish the activity; therefore, the RNA extracts were assayed for T2 antigens. When RNA extracts prepared by these workers or by Fishman were tested, T2 head, tail and internal protein antigens were detected. Since T2 internal protein was found in the extracts and the antigen content of the extracts decreased with prolonged incubation of the T2-macrophage mixture, it seemed that active degradation of the phage by the

macrophages was requisite to an active preparation. Hence, the following explanation of the phenomenon was set forth. The macrophages phagocytize the phage particles, degrade them and attach parts of the antigen to certain low molecular weight RNA's of the cell. Once the RNA-antigen complex enters a lymph node cell, the RNA may function as an adjuvant. The inadequacy of phenol extraction for mRNA and the presence of antigen in the extracts were thought to argue strongly against the "transformation" concept of the mechanism involved.

The idea that RNA-antigen complexes play an important role in the induction and perpetuation of the antibody response was not an entirely new one, for Garvey and Campbell (1957) had shown that various antigens (labelled with radioactive isotopes) were stored in the liver tissue of the recipients in immunogenic form for several months.

Askonas and Rhodes (1964, 1965) studied the induction of antibody formation by RNA extracted from macrophages exposed to I^{131}-labelled Hemocyanin for 2.5 hours <u>in vivo</u> and from macrophages to which the antigen was added immediately before extraction. These extracts were incubated with normal lymph node cells for 1 hour and the mixtures injected into mice primed earlier with the same antigen. The recipients' sera were analyzed for antigen binding capacity at various times after injection. Both extracts were found to be immunogenic. The second type, however, was only about one tenth as active as the first type. Since the addition of antigen to the macrophages immediately before extraction resulted in immunogenic preparations, they concluded that an active process was necessary for RNA-antigen complex formation and that the immunogenecity of the extracts was probably not due to the

formation of specific informational RNA. It was felt 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) incubated RNA extracts of spleen cells from mice immunized with sheep red blood cells (SRBC) with nonimmune spleen cells for 6 days. The system was assayed by the Jerne antibody plaque technique at various intervals. The number of plaques, and thus of antibody producing cells, increased to a maximum at 48 to 72 hours. Pretreatment of the extracts with ribonuclease, but not with deoxyribonuclease, inhibited the increase. Control systems, consisting of nonimmune spleen cells incubated with RNA from nonimmune mice, from mice immunized with chicken erythrocytes or from mice immunized with bovine serum albumin, showed no increase in the numbers of plaques. Thus, the increase in antibody producing cells in the first system was shown to be specific. As in the work of Fishman and Adler (1963), the low molecular weight fraction of the RNA extracts was the active portion. Neither antigen nor antibody was found in the RNA extracts. Friedman (1964) suggested that the RNA extracts might induce antibody formation by: (1) an antibody coding mechanism; (2) an immunogenic antigen-nucleic acid complex; or (3) an antibody precursor. He did not, however, state which mechanism would best account for his results.

In 1964, Cohen and Parks studied a system similar to that of Friedman (1964). In addition to RNA extracts of immune spleen cells, they employed RNA extracts of spleen cells from mice injected with SRBC 3.5 hours earlier and of spleen cells to which antigen was added immediately before extraction. In both of these systems the number of antibody

producing cells was significantly lower than in the immune system. Therefore, Cohen and Parks (1964) postulated that the increase in antibody producing cells after incubation of the active RNA extracts with nonimmune spleen cells could be the result of: (1) an RNA-antigen complex; (2) mRNA "transformation"; or (3) the presence of some factor other than antigen in the RNA.

Further work (Cohen, Newcomb and Crosby, 1965) showed that, whereas treatment with deoxyribonuclease, trypsin, pronase, alpha-amylase or beta-amylase had no effect upon the conversion, treatment of the RNA extracts with ribonuclease or chloramphenicol inhibited the appearance of cells which produced SRBC hemolysins. The active fraction was found to be 8 to 12 S RNA, which corresponds to RNA with a molecular weight of 10^5. When the purified 8 to 12 S RNA was used in the system, the number of converted cells per ug RNA was increased up to 1000-fold; however, the total number of antibody producing cells in a given preparation remained the same. This can be explained, however, as a manifestation of other mechanisms of antibody production, for Jerne (1964) has shown that only a certain number of spleen cells in a given preparation are potentially capable of producing a specific antibody molecule at a given time. In addition, the specific activity of the RNA decreased when RNA from nonimmune animals' spleen cells was used to "dilute" it.

Subsequent studies (Cohen, 1967) showed that the RNA extracts were more resistent to ribonuclease treatment in physiological salt solutions (PSS) than in sodium chloride plus EDTA solutions. In fact, the RNA extracts treated in PSS retained more large molecular weight fragments after ribonuclease treatment than did those treated in the other type

of solution. When minimally digested RNA was added to normal spleen cells, no increase in antibody producing cells was observed. Further, if untreated RNA from immune mice was added to the spleen cells after the addition of minimally digested RNA, only a small increase in the number of antibody producing cells was observed as compared to positive control systems. RNA from spleens of mice immunized with diphtheria toxiod or Escherichia coli did not produce this effect. Cohen (1967) concluded that spleen cells potentially capable of producing SRBC hemolysins have specific recognition sites for the RNA used in these studies as the stimulus for antibody synthesis.

In 1964, Fishman, van Rood and Adler showed, contrary to earlier results, that active RNA preparations could be obtained using T2-macrophage mixtures at a ratio of from 1:1 to 100:1. When diffusion chambers charged with this high antigen input macrophage RNA were inserted into rats, the recipients gave a biphasic response. That is, two peaks of circulating antibody were evident, one at day 4 and another at day 13. These results were contradictory to those of earlier experiments; hence, another experiment was designed to show if there were qualitative differences in RNA capable of stimulating antibody synthesis. The chambers were removed from half the recipients after 16 hours and inserted into other rats. Long-term recipients gave a characteristic biphasic response, short-term recipients gave only the first wave of antibody and secondary recipients of chambers gave only the second wave. Thus it was hypothesized that two types of RNA might be present in these preparations and that the RNA which induced the first wave of antibody might diffuse more quickly than the RNA which induced the second wave.

The characteristic biphasic response was again obtained with in vitro systems and both types of RNA were shown to be dependent upon phage-macrophage interaction. However, the RNA responsible for the second wave of antibody was more refractive to ribonuclease treatment than was the one responsible for the first wave. Experiments using actinomycin D showed the RNA responsible for the first wave to be synthesized upon contact with antigen and the RNA responsible for the second wave to be present before antigenic contact. Further experiments showed that the first wave was 19 S antibody and the second 7 S.

When Adler, Fishman and Dray (1966) stimulated lymph node cells from homozygous b^5 rabbits (the b locus determines the antigenic specificity of the light chains of the antibody molecule) with RNA from macrophages taken from homozygous b^4 rabbits, the first wave of antibody (19 S) had the allotypic specificity of the macrophage donor (b^4), whereas the second wave (7 S) had the specificity of the lymph node cell donor (b^5). The reciprocal experiment gave corresponding results. These results were interpreted as suggesting that at least one of the types of RNA involved in the in vitro induction of antibody synthesis in this system might do so by the mRNA (transformation) mechanism suggested earlier, since only the RNA responsible for the second wave contained antigen.

Ribonuclease-sensitive fractions of lymphoid cells also appear to be involved in the passive transfer of transplantation immunity. In 1962, Mannick and Egdahl found that RNA extracts of lymph node cells from rabbits that had received a skin graft from another type of rabbit could induce transplantation immunity in the graft donor if the RNA was

incubated with lymph node cells from still other types of rabbits and the mixture injected intradermally into the graft donor. When the lymph node cells were injected without being incubated with the RNA, no reaction was seen. Treatment of the RNA extract with ribonuclease inactivated it.

In 1966, Wilson and Wecker studied the same phenomenon with a somewhat different system. Lymphoid cells were removed from DA strain rats that had been sensitized to Lewis strain rat lymphoid cells and RNA extracts were prepared. When nonimmune DA lymphoid cells were incubated with these RNA extracts and then added to cultures of Lewis cells, the DA cells caused the destruction of up to 36% of the target cells within 70 hours' incubation. Significantly smaller percentages of Lewis cells were destroyed in control systems. The degree of destruction of the target cells was generally proportional to the amount of RNA used in pre-incubation of the DA cells and treatment with ribonuclease before incubation inactivated the extracts. These workers tended to support the views of Askonas and Rhodes (1965), as well as those of Friedman, Stavitsky and Solomon (1965), in their interpretation of the possible mechanism involved.

Hashem (1965) demonstrated that RNA extracted from cultures of peripheral lymphocytes stimulated with the antigen to which the donor had been sensitized promoted transformation and mitosis in cultures of autologous unstimulated lymphocytes. The mechanism was ribonuclease-sensitive and specific with respect to the antigen used in the sensitization of the cell donor. This finding has significant implications in regard to both types of immunological phenomena discussed

earlier, since the clonal selection theory of antibody production (Burnet, 1959) involves proliferation and differentiation of competent lymphoid cells as initial steps in the induction of antibody synthesis.

Still another type of immunological phenomenon in which RNA extracts of certain cells have been implicated is the passive transfer of cellular immunity. This phenomenon is usually defined as the acquired intrinsic ability of cells of the reticulo-endothelial system to suppress multiplication of phagocytized organisms.

In 1962, Fong, Chin and Elberg demonstrated passive transfer of cellular immunity against Mycobacterium tuberculosis by the injection of viable histiocytes obtained from immune rabbits into normal recipient rabbits. Cellular immunity was demonstrated in vitro as a lack of cell degeneration when systems composed of immune cells in immune serum, but not normal cells in normal or immune serum, were infected with virulent tubercle bacilli. The recipients' state of cellular immunity was demonstrable by 11 but not by 7 days after injection and could be serially transferred through four sets of recipients. Water lysates of the immune donors' histiocytes were found to confer the same state of immunity upon normal recipients. However, the immunity failed to reach significant proportions until 13 days after injection of immune lysates. Injection of lysates of normal histiocytes caused no change in the recipients' immune status.

In a subsequent study, Fong, Chin and Elberg (1963) investigated the nature of the factor responsible for passive transfer of cellular immunity with immune cell lysates. The transfer factor was found to be stable for at least 24 hours at 25 to 37 C, but was inactivated in 30

minutes at 56 C. Due to the heat-labile nature of the transfer factor and the fact that serial passive transfer of cellular immunity with viable histiocytes had been demonstrated (Fong et al., 1962), the material involved was supposed to be one capable of replication, such as a nucleic acid. Thus, immune lysates were treated with ribonuclease or deoxyribonuclease prior to injection. Ribonuclease, but not deoxyribonuclease, inactivated the transfer factor. The mechanism involved was thus inferred to be RNA-dependent. When various cell fractions of immune lysates were assayed for transfer activity, nuclear and mitochondrial fractions were inactive, whereas ribosomal and microsomal fractions were active. Treatment of microsomal fractions with sodium deoxycholate failed to inactivate this fraction, whereas treatment with ribonuclease inactivated it. Hence, it was concluded that ribosomal RNA was 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 to a lesser degree than were complete cell lysates.

In order to check for the presence of viable M. tuberculosis in their RNA extracts, Fong et al. (1963) injected guinea pigs subcutaneously with a preparation of immune histiocytic ribosomes well in excess of the amount usually used in rabbits. After 4 to 8 weeks, the guinea pigs had no tuberculin skin sensitivity and no complement fixing antibodies specific for M. tuberculosis. Thus, it was concluded that the passive transfer of cellular immunity was due to some sort of replicating RNA from the immune histiocytes and not due to contamination of the preparations with viable bacilli or bacillary antigens. This concept was further supported by the fact that serum from immune animals

was required if the cells were to resist necrotization of tubercle bacilli *in vitro* and by the fact that serial transfer of cellular resistance with viable immune histiocytes was possible (Fong *et al.*, 1962).

Subsequent studies (Fong, Chin and Vickrey, 1963) demonstrated interspecies passive transfer of cellular immunity, with ribosomes from immune histiocytes, between mice and rabbits. In addition, transfer from rabbits to guinea pigs, but not from guinea pig to guinea pig or from guinea pig to rabbit or mouse, was demonstrated. This interspecies transfer was not mediated by a transfer of viable bacilli, since the plating of lysates upon glycerol-blood agar failed to show the presence of viable tubercle bacilli.

The cellular resistance of histiocytes from recipients of immune histiocytic ribosomes was to some degree non-specific, since the recipients' histiocytes were protected from necrotization by *Brucella melitensis*, as well as from that by *M. tuberculosis* (Fong, Chin and Elberg, 1964).

In 1962, Mitsuhashi and Saito reported the successful *in vitro* passive transfer of cellular immunity against *Salmonella enteritidis*. Peritoneal exudate cells obtained from mice immunized with live *S. enteritidis* were planted in bottles. After the cells had settled out, heat-killed *S. enteritidis* organisms were incubated with the cells for 24 hours, the supernatants were decanted and filtered to remove the bacteria. Normal mouse cells were incubated with this supernatant for 72 hours (3 changes), the supernatant was removed and the cultures were inoculated with live *S. enteritidis*. The cells treated with this

supernatant inhibited intracellular multiplication of the organism for at least 3 days, whereas cells treated with supernatants from normal cells or from cells obtained from mice immunized with killed organisms were destroyed within 1 day.

In a subsequent study, Saito and Mitsuhashi (1965) showed that the supernatant obtained from immune cells, as above, was active in a dilution of 1:4 but not in one of 1:8. The active portion of the supernatant (transfer agent) was inactivated by ribonuclease but not by deoxyribonuclease. This transfer agent was non-dialyzable and was inactivated in 24 hours at 37 C. It was concluded that the transfer agent was probably some sort of cellular RNA. Evidently, this RNA was released into the culture medium as a result of exposure to the organism to which the donor mice were immune.

A later study (Sato 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 fractions of these immune monocytes could be destroyed by treatment with ribonuclease but not by treatment with deoxyribonuclease or trypsin. Thus, the transfer agent appeared to be ribosomal RNA, as was the case in the work of Fong et al. (1963).

In 1966, Ostrander passively transferred immunity to Francisella tularensis by the injection of lysates of macrophages from immune rats into normal recipient rats. The cellular resistance was demonstrated in vitro 13 days after lysate injection by the suppression of intracellular multiplication of F. tularensis and the lack of cell destruction

as a result of infection of cell cultures with this organism. Treatment of immune lysates prior to injection with ribonuclease, but not with deoxyribonuclease, abolished the transfer activity of the lysates. Here again, the active portion of immune lysates appeared to be related in some way to RNA obtained from immune cells. In any event, the mechanism of passive transfer of immunity to F. tularensis was shown to be ribonuclease-sensitive.

It was the purpose of the present study to further investigate the role of ribonuclease-sensitive fractions of macrophages from rats immunized with F. tularensis in the induction of immune mechanisms in normal recipients. The modes of in vitro resistance of macrophages from animals immunized with the live organism and of macrophages from animals immunized with immune lysate were compared in an attempt to further elucidate the mechanisms involved in immunity to tularemia. In addition to cellular resistance, an opsonin which appeared in the body fluids of immune lysate recipients was investigated. The relative importance of humoral and cellular factors in the in vitro resistance to F. tularensis was also studied.

MATERIALS AND METHODS

Bacteria

Two strains of *Francisella tularensis*, the attenuated strain, Jap, and the virulent strain, Schu, were used in this study. The original cultures of these organisms were obtained from Dr. Max Moody, Communicable Disease Center, Atlanta, Georgia. These organisms were cultured on glucose cysteine blood agar (GCBA) (Downs *et al.*, 1947). The virulence of Schu was maintained by passage through normal mice. *Salmonella typhimurium* was used as the heterologous organism in the adsorption studies. The culture of this organism was obtained from the bacteriology culture collection, Kansas State Teachers College. This organism was cultured on brain heart infusion agar. In all instances, bacteria used in this study were taken from 24 hour cultures.

Experimental Animals

The rats used in this study were of mixed breeds and were originally obtained from VLK Animal Farm, Marion, Kansas, and from the Lemberger Company, Oshkosh, Wisconsin. White mice, originally obtained from Kansas State University, Manhattan, Kansas, were used in some phases of this study. The white rabbits used in this study were obtained from various local merchants. In all cases, young adult animals were used.

Glassware Preparation

Caution was exercised to maintain a high degree of chemical cleanliness in the preparation of glassware that would ultimately come into contact with tissue culture constituents or bacterial organisms. Prior to use, the glassware was washed in an Alconox solution, rinsed 5 times with tap water, rinsed 5 times with distilled, deionized water and air dried. Glassware was then wrapped, if appropriate, and sterilized along with other heat stable materials by autoclaving at 15 pounds pressure for 15 minutes. After use, the glassware was soaked in a solution of Alconox and Clorox until it was washed. All materials coming into contact with live F. tularensis, however, were placed in a Clorox solution immediately after use and were autoclaved before being handled further, to reduce the hazard of infection with this pathogen.

Standardization of Bacterial Suspensions

The number of viable organisms used in any given experiment was derived by serial dilution of a standard suspension of the organisms. The growth from one slant was suspended in saline and transferred to a spectrophotometer tube. The suspension was then adjusted to read 20% transmission on a Bausch and Lomb Spectronic-20 colorimeter at a wavelength of 600 mu. This was called the standard suspension. In order to assay the number of viable organisms per ml of the standard suspension, serial 10-fold dilutions were prepared in saline, plated onto GCBA and colony counts were determined. The standard suspension was found to contain 5 to 10 X 10^9 viable F. tularensis per ml.

Immunization Procedure

Rats were immunized with a single intraperitoneal injection of 10^6 viable organism of the attenuated strain, Jap. After a 3 week period of observation, they were challenged with an intraperitoneal injection of 10^7 viable organisms of the virulent strain, Schu. Those rats surviving a minimum 3 week observation period after challenge were regarded as immune.

Cell Culture Medium

The cell culture medium used in viable count experiments consisted of Scherer's tissue culture medium containing heparin (1:10,000), 100 units penicillin per ml and 30% (v/v) heated calf serum. Serum was omitted in the medium used for opsonic studied in order to limit the humoral factors present to those harvested from the experimental animals.

Scherer's medium was obtained as a sterile, 10X stock solution from Colorado Serum Company, Denver, Colorado.

Calf serum was prepared in this laboratory using blood, from freshly slaughtered calves, obtained from Fanestil Packing Company, Inc., Emporia, Kansas. After the blood clotted, the fluid was poured off and centrifuged for 15 minutes at 250 g to remove cellular components. The serum was then decanted, sterilized by bacterial filtration and frozen until used. Before incorporation into cell culture medium, the calf serum was heat inactivated at 56 C for 30 minutes.

Cell Harvesting Technique

Animals whose peritoneal macrophages were to be harvested were killed with ether and tacked to a dissection board. The abdominal area was then cleansed with Furacan, the skin laid back and 3 to 5 ml of cell culture medium without calf serum were injected into the peritoneal cavity. The peritoneum was gently massaged to create a cell suspension and was opened along the midline. The exudate was then removed with a capillary pipette.

Lysate Preparation

Lysates were prepared according to the method of Fong et al. (1962). Peritoneal macrophages were harvested from normal and immune rats in Hanks' balanced salt solution containing heparin and penicillin. The cell suspensions were pooled appropriately, the cells washed 2 times in Hanks' solution and counted with a hemacytometer. After a third wash, the cells were resuspended in distilled water to give a concentration of 10^7 cells per ml. The resulting suspensions were incubated in a 37 C water bath for 3 hours and were agitated occasionally. The cell debris was sedimented by centrifugation, the supernatants were decanted and injected intraperitoneally into normal animals. Rats received 2 ml lysate each; mice received 1 ml. Portions of each lysate were plated on GCBA to check for the presence of viable F. tularensis. The lysates were consistently sterile.

Enzyme Treatment of Lysates

Enzyme treatment of lysates was carried out with bovine pancreatic ribonuclease obtained from Mann Research Laboratories, Inc., New York, New York. This commercial preparation was 5 times crystalline, salt-free and without protease activity. The enzyme was dissolved in 0.02 M phosphate buffered saline (pH 7.2) and sterilized by bacterial filtration. It was then heated at 80 C for 10 minutes to remove any contaminating enzyme activity (Fishman and Adler, 1963). A sufficient amount of this solution was then added to the lysates to give a final concentration of 1 mg ribonuclease per ml lysate. This mixture was incubated at 37 C for 20 minutes and then injected into normal recipients.

Viable Count Studies

The exudates from the animals in each group were pooled after harvesting. Cell counts were determined with a hemacytometer, the suspensions were adjusted to 3 X 10^6 cells per ml by the addition of Scherer's and enough heated calf serum to give a final serum concentration of 30%. For controls, 1 ml portions of the uninfected cell suspensions were planted in flying cover slip tubes and incubated in an inclined stationary position at 37 C for the duration of the experiment. In the initial experiments, the remaining suspensions were inoculated with Jap, planted and incubated. In the supernatant exchange experiments, the remaining suspensions were divided in half. The cells in one half of each system were sedimented by centrifugation at 1000 g for 10

minutes and the supernatant fluids were exchanged. The four cell suspensions were then inoculated, planted and incubated as before. These experiments were followed with May-Grüenwald Giemsa stains and plate counts. The pH of the culture medium was adjusted as necessary with 5% sodium bicarbonate in Scherer's.

Plating and Staining Techniques

The cover slips in the tubes to be assayed for viable count were crushed with a glass rod formed to fit the bottom of the tubes. In this way, the cells containing organisms were disrupted and the organisms were released into the medium. Serial 10-fold dilutions were then prepared in saline. Duplicate GCBA plates received 0.1 ml of each appropriate dilution. It was necessary to plate over a range of at least 3 dilutions for each system. The inoculum was spread on the surface of the agar with a sterile bent glass rod. Plate counts were determined after 72 to 96 hours' incubation at 37 C. Since earlier studies (Ostrander, 1966) had shown that F. tularensis did not survive in cell culture medium alone, plate counts were regarded as accurate representations of the number of viable intracellular organisms per tube.

The cover slips in those tubes to be assayed for cell condition were removed and air dried. The cell preparations were fixed in methanol for 5 minutes, stained with May-Grüenwald for 5 minutes, stained with Giemsa for 8 minutes, rinsed in acetone for 1 minute, rinsed in acetone-xylene (1:1) for 1 minute and cleared in xylene for 10 minutes. The stained cover slips were then attached to microscope slides with Permount and observed.

Fluorescent Antibody Preparation

Fluorescent antibody was prepared according to the method of Cherry et al. (1960). A rabbit was immunized with multiple injections of formalin-killed Schu. One week after the last injection, the rabbit was bled from the ear vein. The blood was allowed to clot and the serum collected. The globulins were precipitated at half-saturation with ammonium sulfate overnight in the cold. The mixture was then centrifuged for 30 minutes at 2500 g in the cold, the supernatant discarded and the pellet re-dissolved in distilled water. Precipitation was repeated twice to rid the globulin of hemoglobin. The ammonium sulfate removed by dialysis against 0.85% saline (3 changes) in the cold. The protein content of the globulin was determined by the Biuret method. After adjustment of the solution to 5% protein with saline, 1 ml of carbonate-bicarbonate buffer (pH 9.0) was added for every 9 ml of globulin. The solution was chilled, 0.05 mg fluorescein isothiocyanate per mg protein were added and the mixture was stirred overnight in the cold. The conjugated protein was separated from non-conjugated protein and from unbound fluorescein isothiocyanate on a sephadex G-25 column. The conjugate was eluted from the column with 0.01 M phosphate buffered saline (pH 6.5) and adsorbed with 100 mg anhydrous rat liver powder per ml conjugate to eliminate non-specific staining components. The fluorescent antibody preparation was shown to be specific for F. tularensis by quenching of fluorescence when untagged anti-tularensis serum was added to the smear of organisms prior to staining with

fluorescent antibody and by the lack of staining of heterologous organisms. The preparation was frozen until used.

Opsonic Studies

Peritoneal macrophages were harvested from lysate recipients at appropriate intervals after injection. Cell counts were taken and the concentrations of the separately pooled suspensions were adjusted to 5 X 10^6 cells per ml by the addition of Scherer's. For controls, 1 ml portions of the uninoculated cell suspensions were planted in cover slip tubes. The remaining cell suspensions were inoculated with Jap in an organism to cell ratio of 5 to 1 and planted. The systems were incubated for 3 hours at 37 C in an inclined stationary position. The cover slips were then removed, fixed in acetone for 5 minutes, rinsed in 3 baths of 0.02 M phosphate buffered saline (pH 7.2) for 5 minutes, stained with fluorescent antibody in moisture chambers for 30 minutes, rinsed in 3 baths of phosphate buffered saline and mounted on microscope slides with phosphate buffered glycerol (pH 8.0). The cell preparations were scored with an ultraviolet microscope by counting several hundred cells in randomly selected fields on 3 cover slips and calculating the per cent cells containing organisms.

In supernatant exchange and adsorption experiments, uninoculated cell systems were planted and the cells allowed to attach to the cover slips during 3 hours' incubation at 37 C. The supernatants were then removed and pooled appropriately. For supernatant exchange experiments, the supernatants were inoculated with Jap, replaced on the appropriate cell cultures and the experiments followed as usual. For adsorption

experiments, the supernatants were divided into 3 parts. Two parts were adsorbed with a heavy suspension of F. tularensis or S. typhimurium overnight in the cold. The third part was merely held in the cold overnight. During the interim, the cell cultures were bathed in complete cell culture medium. After the adsorbed supernatants had been filtered to remove the organisms, they were inoculated with Jap and replaced on the cell cultures. The experiments were followed as before.

Agglutination Tests

The rats used in each experiment were bled from the dorsal aorta. The blood was appropriately pooled and allowed to clot. The serum was separated from the clot by centrifugation at 250 g for 15 minutes, decanted and frozen until titered.

The growth from 24 hour cultures of Jap was suspended in 0.85% saline containing 1% formalin and held overnight in the cold. The organisms were then washed with saline 3 times by centrifugation at 2700 g for 20 minutes and the final pellet resuspended in saline to give the standard suspension. The prepared antigen was diluted 1:1 before use in agglutination tests.

Three-tenths ml portions of serial 2-fold dilutions of the serum, lysate or supernatant to be titered were placed in small tubes. An equal amount of prepared antigen was added to each tube, the tubes were shaken and incubated in a 37 C water bath for 3 hours. The tubes were held in the cold overnight before being read for titer.

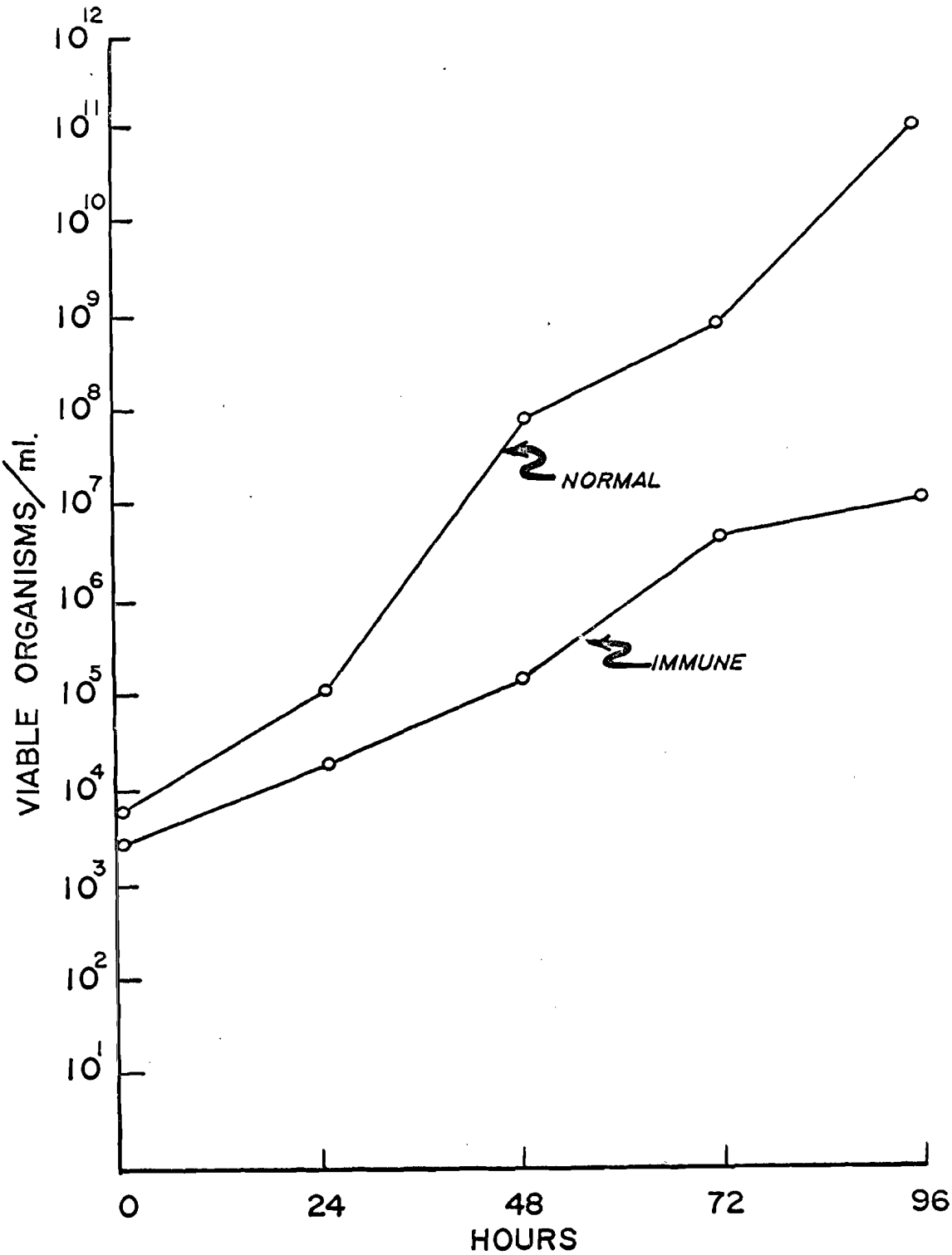
RESULTS

In Vitro Resistance of Cells from Rats Immunized with Viable Organisms

The initial objectives of this study included the confirmation of results reported by Ostrander (1966). Among these results was the observation that peritoneal macrophages from rats immunized with viable F. tularensis could suppress the intracellular multiplication of the organism in an in vitro system. Peritoneal exudate cells were obtained from normal and immune rats, the adjusted cell suspensions inoculated with 10^4 Jap per ml and planted. The experiment was followed with stains and plate counts. The results, based on viable count data, are presented in Fig. 1. The normal cell system allowed extensive proliferation of F. tularensis, whereas the immune cell system suppressed the multiplication of the organism to a large degree. Microscopic observations of stained cover slips confirmed the results obtained from plate counts. Numerous intracellular organisms were evident in the normal cell system at 48 hours after inoculation and by 96 hours the organisms had destroyed most of the cells. In contrast, very few intracellular organisms were evident in the immune cell system at 48 hours and by 96 hours, although intracellular organisms and some cell destruction were evident, most of the cells remained intact. It was concluded that cells from rats immunized with viable F. tularensis were resistant to the organism in the system employed.

Figure 1. Comparison of the multiplication of F. tularensis in normal and immune cell systems.

Figure 1. Comparison of the multiplication of F. tularensis in normal and immune cell systems.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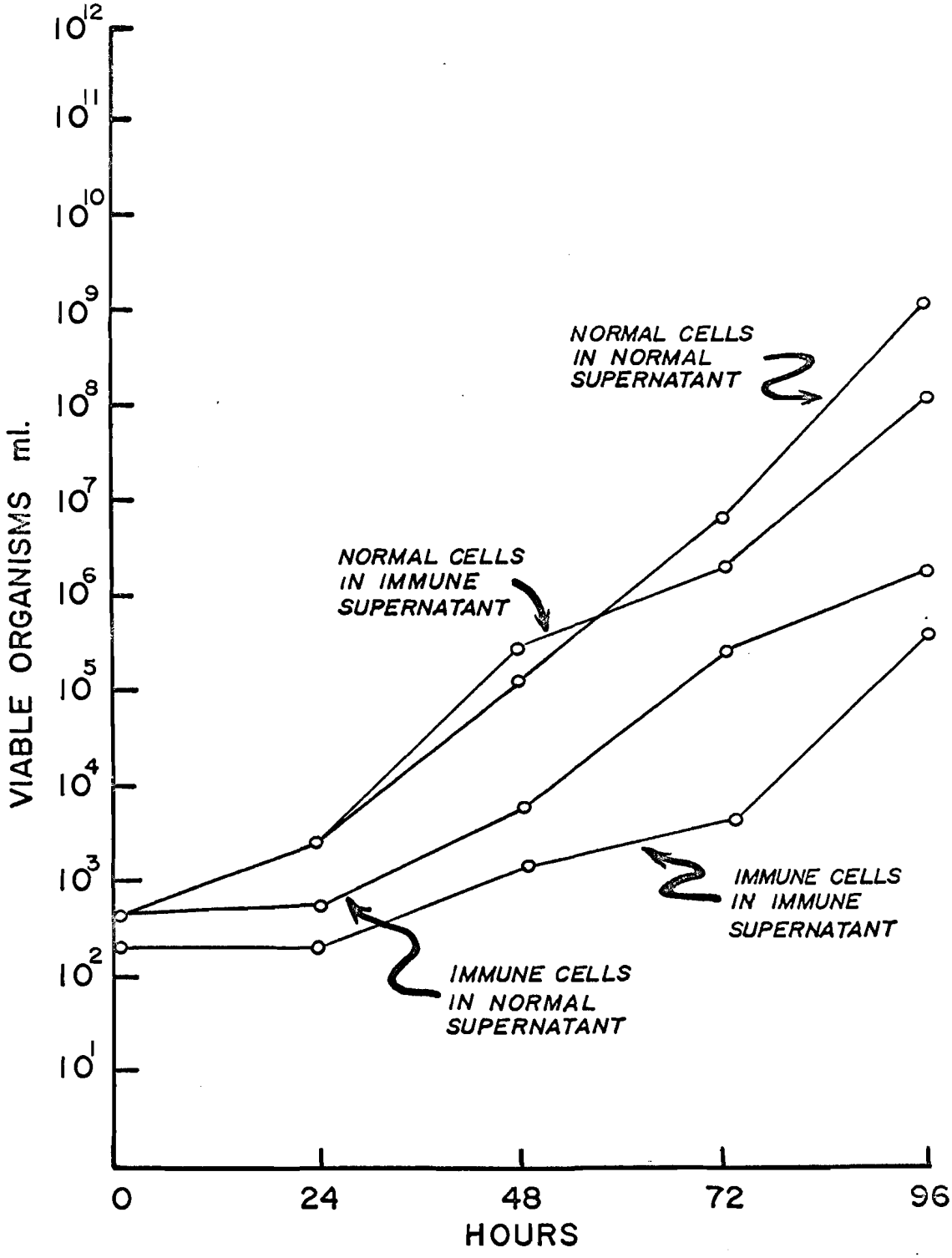
Cellular Immunity in Rats Immunized with Viable Organisms

To determine the relative importance of cellular and humoral factors in resistance to F. tularensis in the cell systems obtained from rats immunized with viable organisms, supernatant exchange experiments were carried out. The cells were separated from their supernatant fraction by centrifugation and resuspended in the desired supernatant to give the following systems: (1) immune cells in immune supernatant; (2) immune cells in normal supernatant; (3) normal cells in immune supernatant; and (4) normal cells in normal supernatant. The cell systems were inoculated with 10^3 Jap per ml and planted. The experiment was followed with stains and plate counts. The most effective control of organismal multiplication was obtained with the complete immune system, i.e., immune cells plus immune supernatant (Fig. 2). The least effective control of organismal multiplication occurred in the complete normal system. However, the fact that immune cells infected and cultured in normal supernatant suppressed the multiplication of the organism, in comparison to normal cells bathed in normal supernatant, was of utmost significance and demonstrated that humoral factors were of secondary importance in resistance to F. tularensis. Microscopic observations again coroborated viable count results. Little cell destruction and few intracellular organisms were observed in immune cell preparations, regardless of the supernatant used in the system, whereas much cell destruction and large numbers of organisms were observed in normal cell preparations by 96 hours after inoculation. These results demonstrated that in vitro resistance to F. tularensis was largely due

Figure 2. Effect of normal and immune humoral factors on the multiplication of F. tularensis in normal and immune cell systems.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NORMAL CELLS IN NORMAL SUPERNATANT
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IMMUNE CELLS IN NORMAL SUPERNATANT
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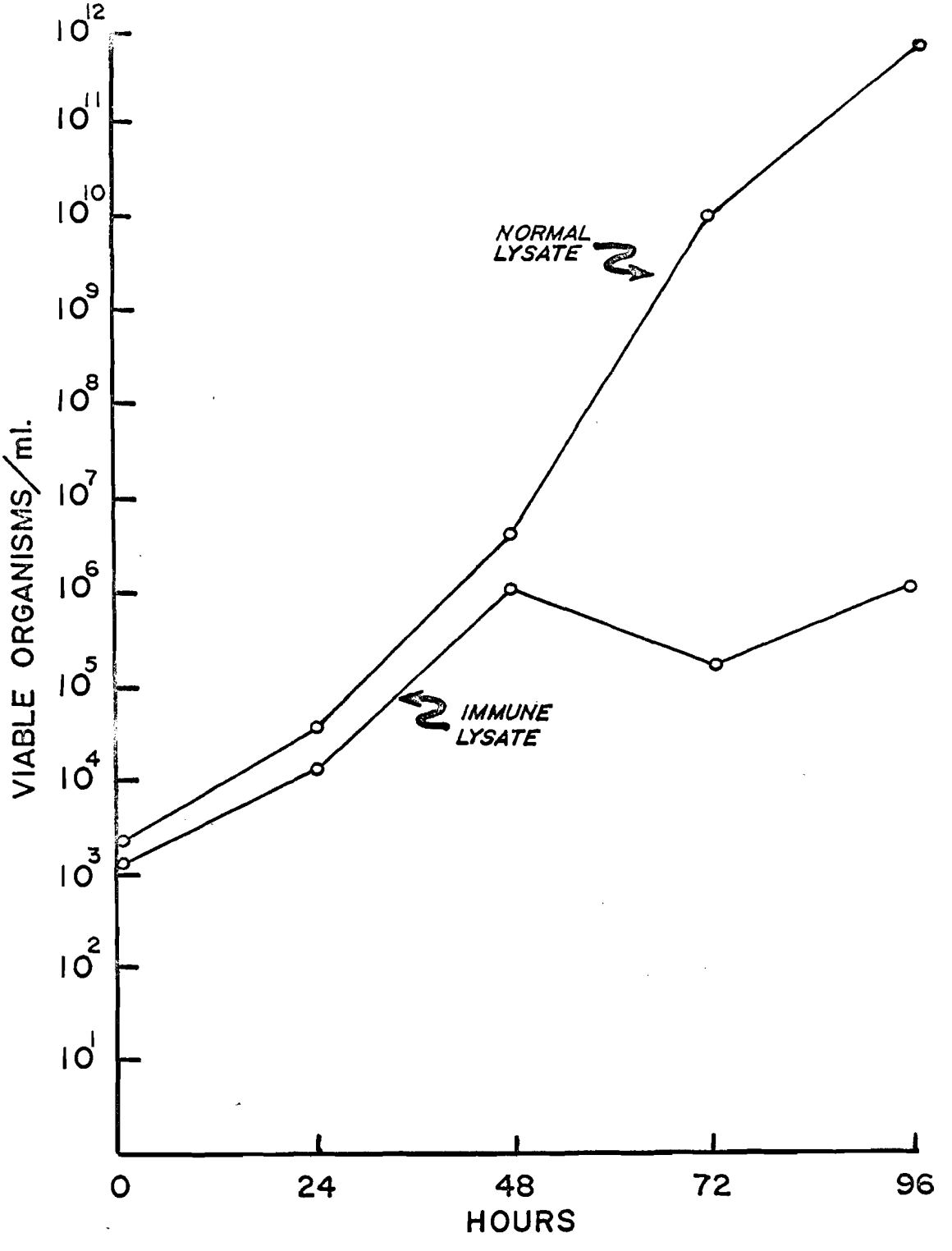
HOURS

to cell-associated factors and that humoral factors were of secondary importance.

In Vitro Resistance of Cells from Immune Lysate Recipient Rats

Among the results reported by Ostrander (1966) that were to be confirmed in the initial phases of this study was a report of passive transfer of immunity to F. tularensis by the injection of lysates of macrophages from immune rats into normal rats. Normal and immune cell lysates were prepared and injected into normal rats. After 13 days, cell suspensions were obtained from the lysate recipients and viable count experiments set up. Because these experiments were intended to determine only if immune mechanisms could be passively transferred to normal animals by injections of immune cell lysates, the cells were not separated from their attendant supernatant fluid. The pooled cell suspensions were adjusted, inoculated with 10^4 Jap per ml, planted and the experiment was followed as before. Results presented in Fig. 3 indicate that cell systems obtained from immune lysate recipients were more effective in the suppression of intracellular multiplication of F. tularensis than were cell systems obtained from normal lysate recipients. Here again, microscopic examination of cover slips supported results obtained from viable count data. By 96 hours post-infection, the organisms were quite evident and had virtually destroyed the cells in the normal lysate cell system, whereas they were evident in small numbers and had caused only a slight degree of cell destruction in the immune lysate cell system. It was apparent that immune mechanisms could, indeed, be passively transferred to normal rats with

Figure 3. Comparison of the multiplication of F. tularensis in normal lysate and immune lysate cell systems.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NORMAL LYSATE
IMMUNE LYSATE
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HOURS

immune cell lysates and that normal cell lysates caused no detectable change in the recipients' immune status.

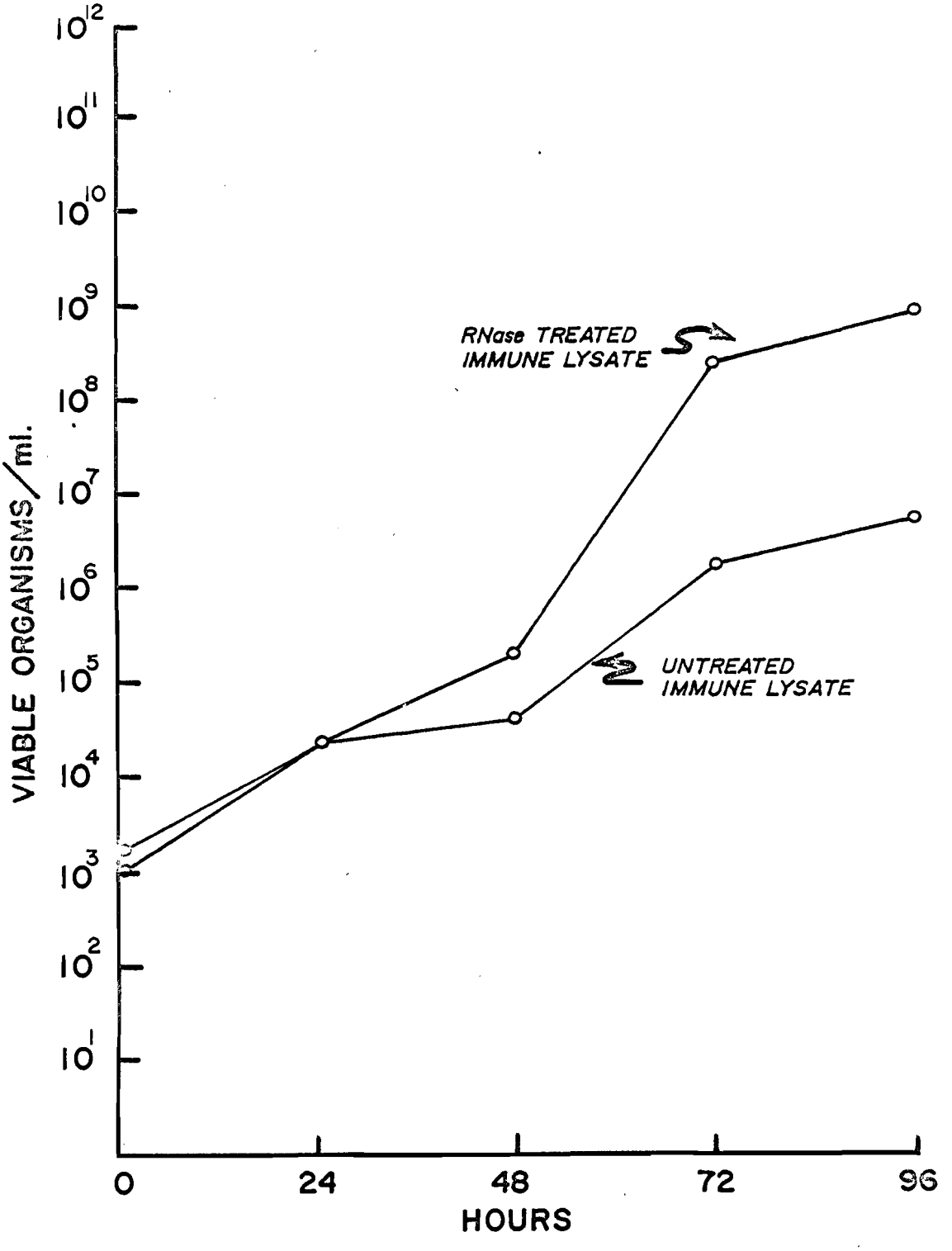
Ribonuclease-Sensitivity of Passive Transfer of Resistance

The passive transfer of resistance reported by Ostrander (1966) was destroyed when immune cell lysates were treated with ribonuclease prior to injection. Therefore, immune cell lysates were prepared and divided into 2 portions; 1 portion was treated with ribonuclease and both portions were injected into normal rats. Thirteen days later, cell suspensions were obtained from the recipients and a viable count experiment set up. The adjusted cell suspensions were inoculated with 10^4 Jap per ml, planted and the results followed as usual. Cell systems derived from recipients of ribonuclease-treated immune lysates failed to suppress intracellular multiplication of the organism as compared to cell systems from recipients of untreated immune lysates (Fig. 4). Microscopic observations were in accord with results obtained from viable counts. It was concluded that passive transfer of immunity to F. tularensis with immune cell lysates was mediated by a ribonuclease-sensitive mechanism.

Appearance of Opsonic Activity in Immune Lysate Recipient Rats

Ribonuclease-sensitive fractions of macrophages exposed to antigen have been implicated in the induction of antibody production (Friedman, 1964; Askonas and Rhodes, 1965; Friedman et al., 1965; Adler et al., 1966; Cohen 1967). Since the appearance of antibody in immune lysate recipients, whether it resulted from passive or active mechanisms, could account solely, or at least in part, for the resistance observed in cell

Figure 4. Comparison of the multiplication of F. tularensis in ribonuclease-treated immune lysate and untreated immune lysate cell systems.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IMMUNE LYSATE
UNTREATED
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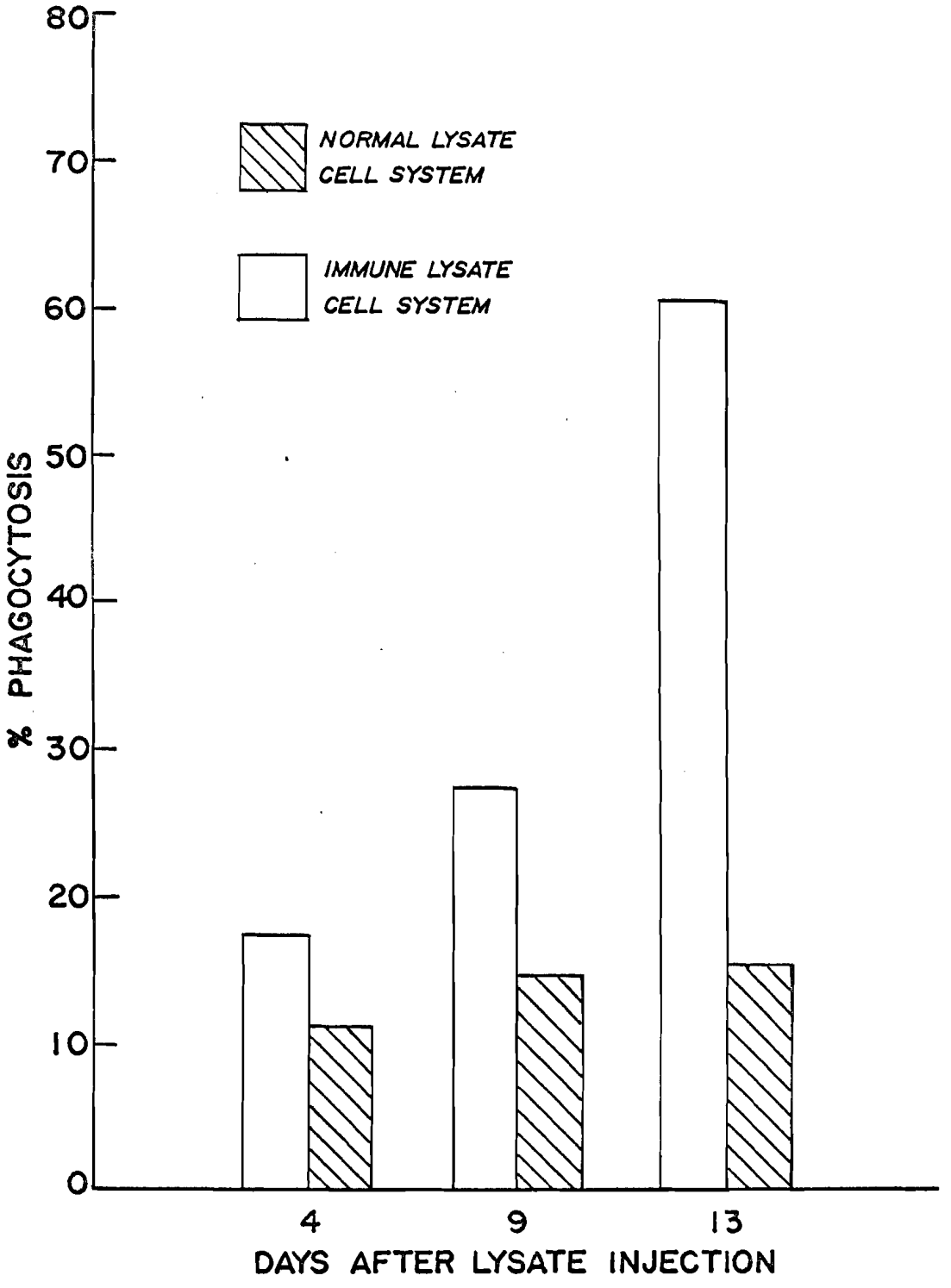
HOURS

systems obtained from immune lysate recipients, agglutination tests were performed on (1) immune lysates prior to injection, (2) immune lysate recipients' sera and (3) immune lysate recipients' peritoneal fluids. When these tests failed to reveal antibody, more sensitive opsonic studies were employed. Cell suspensions were harvested from normal and immune lysate recipients 4, 9 and 13 days after injection. The adjusted cell suspensions were inoculated with Jap in a 5 to 1 organism to cell ratio, planted and incubated for 3 hours at 37 C. Cover slips were removed, stained with fluorescent antibody and the per cent phagocytosis was determined. The results presented in Fig. 5 demonstrate a temporal increase in the per cent phagocytosis in immune lysate cell systems from 18% on day 4 to 60% on day 13. No corresponding increase was seen in normal lysate cell systems. It was evident that opsonic activity appeared in the peritoneal fluids of immune lysate recipients and that the activity increased for at least 13 days after injection.

Ribonuclease-Sensitivity of Appearance of Opsonic Activity

With the appearance of opsonic activity in the peritoneal fluids of immune lysate recipients, it became important to determine if this immune mechanism was also due to ribonuclease-sensitive fractions of the immune cell lysates. Lysates were prepared from immune and normal cell pools, half of each lysate was treated with ribonuclease and the lysates were injected into normal animals. Thirteen days later, cell suspensions were obtained from the recipients, adjusted, inoculated with 5 Jap per cell and planted. After 3 hours' incubation at 37 C, cover slips were removed, stained with fluorescent antibody and the per cent

Figure 5. Demonstration of sequential increase of opsonic activity in immune lysate recipients.


80
70
60
50
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20
10
0
% PHAGOCYTOSIS
NORMAL LYSATE CELL SYSTEM
IMMUNE LYSATE CELL SYSTEM
4
9
13
DAYS AFTER LYSATE INJECTION

phagocytosis was determined. The per cent phagocytosis in the cell system derived from recipients of ribonuclease-treated immune lysate was nearly the same as that in the system from normal lysate recipients, but only about one-third as large as that in the system derived from recipients of untreated immune lysate (Table I). The decrease in opsonic activity was not due to adverse effects of the enzyme upon the macrophages, since the per cent phagocytosis in the system derived from recipients of ribonuclease-treated normal lysate was nearly the same as that in the normal lysate control system. It was concluded that the appearance of opsonic activity, as well as the induction of resistance, in immune lysate recipients was the result of a ribonuclease-sensitive mechanism.

Association of Opsonic Activity with the Supernatant Fraction of Peritoneal Exudates of Immune Lysate Recipients

Since numerous non-specific substances are known to enhance phagocytic efficiency, steps were taken to characterize the factor responsible for the opsonic activity that appeared in the peritoneal fluids of immune lysate recipients. The first step in this characterization was to determine whether the substance responsible for the activity was associated with the cellular or with the supernatant fraction of the exudate. Cell suspensions were obtained from normal and immune lysate recipients 13 days after injection, the cell counts adjusted and the uninoculated cell suspensions planted. When the cells had settled out and attached to the cover slips, the supernatant fluids were removed, appropriately pooled, inoculated with Jap in an organism

Table 1. Effect of ribonuclease treatment of lysates prior to injection.

ORIGIN OF PHAGOCYTIC SYSTEM	TREATMENT OF LYSATE PRIOR TO INJECTION	PER CENT PHAGOCYTOSIS
Immune Lysate Recipients	None	52
Immune Lysate Recipients	Ribonuclease	18
Normal Lysate Recipients	None	17
Normal Lysate Recipients	Ribonuclease	16

to cell ratio of 5 to 1 and replaced on the cell cultures to give the following systems: (1) immune lysate cells in immune lysate supernatant; (2) normal lysate cells in immune lysate supernatant; (3) immune lysate cells in normal lysate supernatant; and (4) normal lysate cells in normal lysate supernatant. Additional systems, in which the inoculum was added to the cell cultures in fresh Scherer's medium, were set up. Three hours later, cover slips were removed, stained with fluorescent antibody and the per cent phagocytosis was determined. The system composed of normal lysate cells bathed in immune lysate supernatant approached the complete immune lysate cell system in phagocytic efficiency, whereas the phagocytic efficiency of the system composed of immune lysate cells and normal lysate supernatant was similar to that of the complete normal lysate cell system (Table II). Neither cell system which received the inoculum in fresh Scherer's medium demonstrated a high degree of opsonic activity. Therefore it was concluded that the factor responsible for the opsonic activity that appeared in immune lysate recipients was associated with the supernatant fraction, rather than with the cellular fraction, of the exudates obtained from these animals.

Globulin Nature and Specificity of Opsonin

Because the opsonic activity was associated with the supernatant fraction of the recipients' peritoneal exudates, the factor responsible for the activity was presumed to be humoral in nature. Most antibodies are associated with the globulin fraction of serum. The supernatant fraction of exudates from immune lysate recipients was treated with

Table II. Association of opsonic activity with the supernatant fraction of peritoneal exudates from immune lysate recipients.

ORIGIN OF PHAGOCYTIC SYSTEM		PER CENT
MACROPHAGES	SUPERNATANT	PHAGOCYTOSIS
Immune Lysate Recipients	Immune Lysate Recipients	59
Normal Lysate Recipients	Immune Lysate Recipients	49
Immune Lysate Recipients	Normal Lysate Recipients	23
Normal Lysate Recipients	Normal Lysate Recipients	19
Immune Lysate Recipients	Fresh Scherer's	10
Normal Lysate Recipients	Fresh Scherer's	7

half-saturated ammonium sulfate, the resulting precipitate reconstituted and dialyzed to remove the ammonium sulfate. This globulin preparation was then added to fresh Scherer's medium to give approximately the original concentration, the mixture inoculated with Jap and added to normal cell cultures. After 3 hours, the per cent phagocytosis was determined as usual. The addition of this preparation to normal cell cultures increased the phagocytic efficiency by a factor of 3 over that observed in the system composed of normal cells bathed in normal supernatant. It thus appeared that the opsonin was found in the globulin fraction and that it might be antibody.

If the opsonin were antibody, it would be specific for the homologous organism, F. tularensis, but not for an unrelated organism. Hence, adsorption studies were designed to establish the specificity of the opsonin. Cell suspensions were obtained from immune lysate recipients 13 days after injection. The uninoculated cell suspensions were adjusted and planted in cover slip tubes. When the cells had attached to the cover slips, the supernatant fluids were removed, pooled and adsorbed. One aliquot of the supernatant was adsorbed with F. tularensis, another with S. typhimurium and the third was left untreated. After the organisms had been removed from the adsorbed supernatants by filtration, the supernatants were inoculated with Jap as before and replaced on the cell cultures. Cover slips were removed three hours later, stained with fluorescent antibody and the per cent phagocytosis was determined. The results presented in Table III demonstrate that adsorption with the homologous organism, F. tularensis, but not with a heterologous organism, S. typhimurium, abolished the opsonic activity.

Table III. Demonstration of opsonin specificity for *F. tularensis*.

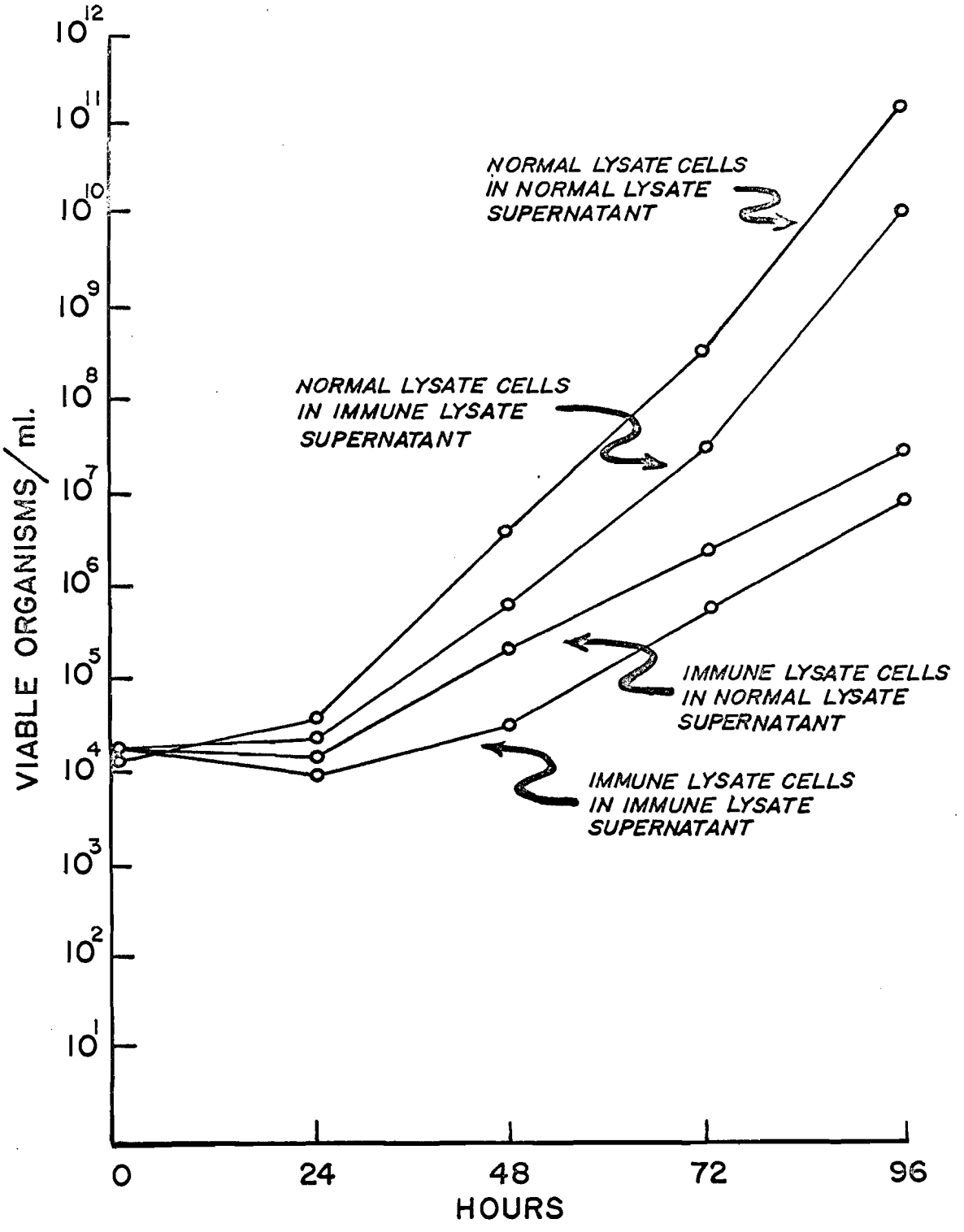
ORIGIN OF PHAGOCYTIC SYSTEM	SUPERNATANT TREATMENT	PER CENT PHAGOCYTOSIS
Immune Lysate Recipients	None	59
Immune Lysate Recipients	*F. tularensis* Adsorption	10
Immune Lysate Recipients	*S. typhimurium* Adsorption	59

These results indicated that the opsonin was specific for F. tularensis and that it was probably an antibody. In addition, the earlier conclusion that the opsonin was associated with the supernatant fraction of peritoneal exudates from immune lysate recipients was more clearly established.

Cellular Immunity in Rats Injected with Immune Lysate

The appearance of a specific opsonin in immune lysate recipients necessitated the determination of the relative importance of humoral and cell-associated factors in the *in vitro* resistance to F. tularensis of cell systems derived from these animals. To this end, supernatant exchange experiments were performed. Cell suspensions from normal and immune lysate recipients were adjusted, the supernatant fraction separated from the cells by centrifugation and the cells resuspended in the desired supernatant to give the following systems: (1) immune lysate cells in immune lysate supernatant; (2) immune lysate cells in normal lysate supernatant; (3) normal lysate cells in immune lysate supernatant; and (4) normal lysate cells in normal lysate supernatant. The cell systems were inoculated with 10^4 Jap per ml, planted and the experiment was followed with stains and plate counts. The most effective suppression of organismal multiplication was obtained with the complete immune lysate cell system; the least effective suppression with the complete normal lysate cell system (Fig. 6). The system composed of immune lysate cells infected and cultured in the presence of normal lysate supernatant, however, exhibited significant control of organismal multiplication as compared to the system composed of normal lysate cells

Figure 6. Effect of normal lysate and immune lysate recipients' humoral factors on the multiplication of F. tularensis in normal lysate and immune lysate cell systems.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NORMAL LYSATE CELLS IN NORMAL LYSATE SUPERNATANT
NORMAL LYSATE CELLS IN IMMUNE LYSATE SUPERNATANT
IMMUNE LYSATE CELLS IN NORMAL LYSATE SUPERNATANT
IMMUNE LYSATE CELLS IN IMMUNE LYSATE SUPERNATANT
0
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HOURS

in immune lysate supernatant. Microscopic observations corroborated the results obtained from plate count data. Extensive cell degeneration and large clusters of F. tularensis were evident in the normal lysate cell systems by 96 hours post-infection, whereas little cell destruction and few clusters of organisms were evident in the immune lysate cell systems. These results indicated that the injection of immune cell lysates into recipients resulted in the induction of cellular immunity as well as in the appearance of a specific opsonin. It was concluded that cell-associated factors constituted the basis of in vitro resistance to F. tularensis in immune lysate cell systems and that humoral factors were of secondary importance only.

Lack of Opsonin Appearance in Mice Injected with Lysates of Immune Rat Macrophages

The following experiments were performed in an effort to elucidate the mechanisms involved in the passive transfer of immune mechanisms to normal animals by the injection of immune cell lysates. Although the presence of antigen in the form of viable F. tularensis in immune lysates was ruled out by a lack of growth of the organism when immune lysates were plated on GCBA, non-viable antigen could have been contained in these preparations. Therefore, precipitin tests, using immune lysate as precipitinogen and anti-tularensis serum of known titer as precipitin, were performed on immune lysates before injection. The tests were uniformly negative. Since the precipitin test is not highly sensitive, another type of experiment was designed to test for the presence of antigen in immune lysates. These experiments were based

upon the idea that if specific antigen were contained in immune lysates and if the ribonuclease-sensitive portion of the transfer factor had nothing to do with the specificity of the transfer, then successful interspecies passive transfer of immune mechanisms with immune lysates would demonstrate the presence of antigen in these preparations. Opsonic studies were used to assay the development of immune mechanisms since the opsonin had been demonstrated to be present even when the degree of *in vitro* resistance of the recipients' cells was not significant in rat to rat transfers. Lysates were prepared from immune rat macrophages and injected into normal mice. Thirteen days later, peritoneal cells were harvested from the lysate recipients and from normal mice. The adjusted cell suspensions were inoculated with Jap in a 5 to 1 organism to cell ratio and planted. After 3 hours' incubation, cover slips were stained with fluorescent antibody and the per cent phagocytosis was determined. The results presented in Table IV indicate that an opsonin did not appear in mice injected with lysates prepared from immune rat macrophages. Therefore, the passive transfer of immune mechanisms appeared to be species-specific and no far-reaching conclusions concerning the mechanisms involved could be drawn.

Table IV. Demonstration of species-specificity of passive transfer of immune mechanisms with immune cell lysates.

ORIGIN OF PHAGOCYTIC SYSTEM	PER CENT PHAGOCYTOSIS
Mice which Received Lysates of Immune Rat Macrophages	9
Normal Mice	6

DISCUSSION

The results obtained in the present study lead to the following conclusions. (1) Peritoneal macrophages from rats immunized with viable F. tularensis possess unidentified factors which allow them to suppress the intracellular multiplication of [illegible]. (2) These factors are cell-associated because the macrophages are effective in the absence of specific antibody. (3) Whereas humoral factors enhance the control of organismal multiplication, they are effective only to a limited extent in resistance to the organism and therefore appear to be of secondary importance in relation to cellular immunity. (4) Water lysates of immune macrophages are capable of passively transferring resistance against the homologous organism, F. tularensis, upon injection into normal rats. (5) The mechanism of this passive transfer is ribonuclease-sensitive. (6) In addition to resistance as demonstrated in vitro, ribonuclease-sensitive fractions of immune cell lysates induce the formation of a specific opsonin in normal recipients. (7) The opsonin is associated with the globulin fraction of the supernatant fluids of peritoneal exudates from immune lysate recipients and appears to be antibody, even though it could not be detected with standard agglutination tests. (8) The role of the opsonin in the in vitro resistance of cell systems derived from immune lysate recipients is secondary in relation to cellular immunity. (9) Finally, the passive transfer of immune mechanisms with immune cell lysates appears to be species-specific.

In 1962, Allen demonstrated passive transfer of immunity against F. tularensis to normal mice by the injection of viable peritoneal cells obtained from mice immunized with the viable organism. The degree of resistance conferred upon recipients was proportional to the number of immune cells injected. Further, immune serum was shown to be ineffective in the passive transfer of resistance. Therefore, Allen (1962) concluded that immunity to F. tularensis was due to an altered state of the tissues.

Stansberry and Woodward (1962) reported a similar passive transfer of immunity to tularemia. They noted that a critical minimal number of viable cells had to be injected to confer significant resistance upon recipients, but failed to detect any relationship between the number of cells injected and the degree of resistance attained when larger numbers of cells were used. They also concluded that immunity to F. tularensis resulted from cellular, rather than humoral, mechanisms of host resistance.

Thorpe and Marcus (1964a) reported 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. In a later paper (Thorpe and Marcus, 1964b), it was 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 are of primary importance and that humoral factors are of secondary importance in immunity to F. tularensis.

It is apparent that the results of Allen (1962), Stansberry and Woodward (1962) and Thorpe and Marcus (1964b) were essentially the same as those obtained in the initial phases of the present study. It would seem, then, that the importance of cellular factors far outshadows that of humoral factors in resistance to the facultative intracellular parasite, F. tularensis.

Although Allen (1962) and Stansberry and Woodward (1962) were successful in passively transferring immunity to tularemia with viable cells from immunized donors, transfers using homogenates of the cells failed. In the present study, water lysates of immune cells were shown to be capable of conferring cellular resistance upon normal recipients. Perhaps the disparity in results is due to the method used in the preparation of disrupted cells for the transfer. Both Allen (1962) and Stansberry and Woodward (1962) used a freeze-thaw method, whereas a much milder form of cell disruption (lysis in distilled water) was used in the present study. Another possibility might be in the method of assaying for resistance to the organism. An in vitro cell system inoculated with attenuated organisms was used in the present study, whereas in the other two studies, recipients of immune cell homogenates were assayed for immunity in vivo, by challenge with the virulent strain of the organism.

With the demonstration of in vitro resistance to F. tularensis in cell systems derived from immune rats and the demonstration of passive transfer of resistance to normal recipients with ribonuclease-sensitive

fractions of immune cell lysates, the present study has confirmed some of the more pertinent results reported by Ostrander (1966). Results obtained in other phases of the present study have extended the knowledge gained from these earlier observations.

The passive transfer of resistance to *M. tuberculosis* reported by Fong *et al.* (1963) was similar to the *in vitro* transfer of resistance to *S. enteritidis* reported by Sato and Mitsuhashi (1965). In both of these studies, the transfer factor was associated with the ribosomal fraction of immune cells, was ribonuclease-sensitive and was, therefore, thought to be ribosomal RNA. However, the resistance observed by Fong *et al.* (1963) was mediated by a non-specific factor contained in immune serum, whereas that observed by Sato and Mitsuhashi (1965) was not affected by the removal of immune serum.

Although cellular immunity in their system was mediated by humoral factors, Fong *et al.* (1963) failed to detect a humoral response in recipients of ribosomal fractions of immune cells. In contrast, the cellular resistance in the system used in the present study was enhanced by, but not dependent upon, humoral factors and a specific opsonin was detected in the peritoneal fluids of immune lysate recipients. This opsonin was a specific recipient response to the injection of ribonuclease-sensitive fractions immune cell lysates because (1) it did not appear in normal lysate recipients, (2) it did not appear when the immune lysate was treated with ribonuclease prior to injection, (3) it increased in titer with time after injection of immune lysates and (4) it was removed from the peritoneal exudate by adsorption with the

homologous organism, F. tularensis, but not by adsorption with the heterologous organism, S. typhimurium.

The opsonin does not appear to play a major role in the in vivo resistance of cell systems derived from immune lysate recipients; however, the fact that cellular immunity and antibody production can be simultaneously induced by ribonuclease-sensitive fractions of immune cells is of importance. It is not known whether or not both immune mechanisms are induced by the same portion of these fractions of immune cell lysates. In this regard, both of the other reports of passive transfer of cellular immunity with portions of immune cells (Fong et al., 1963; Sato and Mitsuhashi, 1965) have implicated ribosomal RNA as the active factor, whereas the reports of induction of antibody with extracts of cells exposed to antigen (Fishman and Adler, 1963; Friedman, 1964; Cohen et al., 1965) have implicated low molecular weight RNA as the active factor. With this as a basis, one would expect two active substances to be contained in the ribonuclease-sensitive fractions of immune cell lysates used in the present study. This idea is not totally without precedent, since Adler et al. (1966) reported two types of RNA present in macrophage RNA from high antigen input systems, each of which induced a different type of antibody in normal lymph node cells. These two types of RNA evidently induced antibody production by a somewhat different mechanism, since one of them was antigen-free and was synthesized upon antigenic contact, whereas the other one contained antigen and was found to be present in the macrophages before antigenic contact (Fishman et al., 1964).

The exact nature of the ribonuclease-sensitive fractions of immune cell lysates responsible for the passive transfer of immune mechanisms is unknown. Therefore, the mechanisms involved in the induction of cellular immunity and opsonin production remain obscure. The appearance of a specific opsonin in immune lysate recipients, however, suggests antigenic stimulation. This hypothesis is further supported by the observation that the opsonin increased in titer with time up to 13 days after injection of immune lysate, since this temporal relationship is characteristic of a primary response to an antigen. The passive transfer of immune mechanisms appears to be species-specific. This and the RNA-dependent nature of the mechanism involved in the induction of these mechanisms in immune lysate recipients indicates that, if antigen is present, it is probably attached to, and inactive without, the ribonuclease-sensitive material. The ribonuclease-sensitive material is probably some form of RNA. One reason for the inactivity of the hypothetical free antigen may be that the RNA portion of the RNA-antigen complex acts both as a carrier for the antigen and as a molecule which enhances the uptake of the antigen by the pertinent cells of the recipient. This concept is similar to that proposed by Friedman et al. (1965) and by Askonas and Rhodes (1965). As proposed by Cohen (1967) the RNA portion of the hypothetical complex may bond to specific receptor sites on the surface of the pertinent cells and thus ensure the preferential uptake of the particular complex.

The "transformation" concept of the mechanism involved in the passive transfer of cellular resistance, as proposed by Fo[illegible] et al.

(1963), cannot be discarded, however, until specific antigen is demonstrated in immune cell lysates. As indicated earlier, the transfer of immune mechanisms described in this study may be the result of two separate portions of the ribonuclease-sensitive fractions of immune cell lysates. As such, the portion which induces the opsonin may consist of an RNA-antigen complex and may operate by the corresponding mechanism proposed earlier; the portion which induces cellular immunity may consist of high molecular weight RNA and may operate by the mRNA "transformation" mechanism.

The elucidation of the nature of the factor, or factors, responsible for the passive transfer of immune mechanisms with immune cell lysates must be accomplished before the mechanisms involved can be clearly established. This may be accomplished in part by the separation of the two immune mechanisms induced by the immune cell lysates. Such a separation could be effected by density gradient centrifugation if the transfer factors are of different molecular weights. Preliminary experiments have indicated that such a separation might also be effected by the use of animals immunized with killed F. tularensis as donors of cells for lysates. It has been observed that opsonin formation, but not cellular immunity, may be induced in recipients of such lysates. Experiments in which immune cell lysate recipients are treated with actinomycin D may also furnish insight into the mechanisms involved in the passive transfer demonstrated in the present study. Since actinomycin D inhibits the DNA-dependent RNA-polymerase synthesis of RNA (Goldberg and Reich, 1964), one would expect the administration of this antibiotic to immune cell lysate recipients to delay, at the very least,

the development of an immune response which is mediated by a mechanism that requires the synthesis of mRNA, as would be the case with the Ab-antigen complex concept of the mechanism involved.

SUMMARY

Cell systems derived from rats immunized with viable F. tularensis were able to suppress intracellular multiplication of this organism in vitro. This in vitro resistance was based primarily upon cellular immunity rather than upon antibody. The injection of water lysates of macrophages from rats immunized with viable organisms effected a passive transfer of similar immune mechanisms to normal recipients, whereas the injection of lysates of macrophages from normal rats had no effect. The transfer was found to be mediated by a ribonuclease-sensitive mechanism, as shown by the failure of ribonuclease-treated immune lysates to induce resistance in normal recipients. In addition, the formation of an opsonin was induced by ribonuclease-sensitive fractions of immune cell lysates. The opsonin was specific for F. tularensis, as shown by selective adsorption by this organism and by a lack of adsorption by S. typhimurium. The in vitro resistance of cell systems derived from immune lysate recipients was due primarily to cellular immunity and secondarily to the opsonin. The passive transfer of immune mechanisms with ribonuclease-sensitive fractions of immune cell lysates was shown to be species-specific by the lack of opsonin formation in mice injected with lysates prepared from immune rat macrophages. It would appear, then, that certain fractions of macrophage RNA play a definite role in the initiation of antibody formation in vivo, as well as in vitro, and in the initiation of cellular immunity.

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LITERATURE CITED

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