THE KINETICS OF ANTIBODY PRODUCTION IN MICE TO THE DD34 STRAIN OF PSITTACOSIS

A Thesis Presented to
the Department of Medical Microbiology
The University of Manitoba

In Partial Fulfillment
of the Requirement for the Degree of
Master of Science

by
Sami Faez Muhtadie

August, 1971

ACKNOWLEDGEMENT

This study was carried out under the supervision of Dr. H. Sayed of the Department of Medical Microbiology, whose continual guidance and encouragement is sincerely appreciated. The author would like to convey his appreciation to Dr. J.C. Wilt, head of the Department of Medical Microbiology, for his constructive criticism and suggestions. Grateful acknowledgement is offered to Drs. H.L. O'Brien and J. Hoogstraten of the Children's Hospital for their help in the pathological studies.

A special and sincere thanks go to Dr. F. Chebib of the Faculty of Dentistry for his help in the preparation of this manuscript.

ABSTRACT

The kinetics of antibody production in mice to DD34 strain of psittacosis was investigated using different serological and immunobiological techniques. CF_1 and BALB/C strains of mice were injected intraperitoneally and in the foot pads, and were infected orally. The sera collected over the span of two months were analyzed by the complement fixation, hemagglutination inhibition, immunoelectrophoresis, passive hemagglutination, passive cutaneous anaphylaxis and infectivity neutralization techniques. In addition, the presence of cellular immunity was assessed using the macrophage spreading inhibition technique.

The results of these investigations indicate that both the complement fixation and hemagglutination inhibition techniques may be adequately used at the early stage of immunization. Although the hemagglutination inhibition technique was found to be the more sensitive technique, the results suggested the superiority of the complement fixation technique at later stages of immunization. The results of immunoelectrophoresis study indicated the presence of the complement fixing antibodies in both the 7S and 19S protein fractions of immunoglobulin. Allergic antibodies which contribute to skin reactions were found to be present using the passive cutaneous anaphylaxis technique. Protective antibodies were found present one month after immunization

using the infectivity neutralization technique.

The possible application of these results to the diagnosis of human psittacosis infections was discussed.

TABLE OF CONTENT

INTRODUCTION

Chlamydial infections in humans and animals cause various types of diseases such as pneumonitis, inclusion conjunctivitis and venereal diseases. Several studies have been conducted with the objective of studying the immune response to the chlamydial agents (Rivers et al. 1930, Meyer and Eddie 1962, Collier, 1966). Most of these studies, however, have been restricted to experimental investigations in which one or more serological tests were utilized at one time during the active course of the disease. No attempt has been made to relate the immune response of the host to the chlamydial agent at various stages of the disease. Also no search was made in order to understand the kinetics of production of antibodies which may appear during the course of infection and which are detected by different serological or immuno-biological tests.

It is well recognized that several serologically active antibodies to the chlamydial agents appear during the course of infection (Hilleman et al. 1951, Meyer and Eddie 1962, Reeves and Graham 1962, Sayed and Wilt 1971). It is not known, however, which of these antibodies appear after the infection ie: the Complement fixing, the Hemagglutination inhibition, the infection neutralization, and the passive cutaneous anaphylaxis. Nor has there been any investigation about the behaviour of the cellular-mediated immunity after

infection with chlamydial agents.

Determination of a specific serological test to be used for diagnosis of a chlamydial infection depends to a great extent on the knowledge of the serological activity of antibodies which might appear singly or together.

The degree of potency of the antibody in combating and neutralizing the infection in man, has been given little concern. An investigation of the neutralizing activity of the antibody during the course of infection would provide valuable information on the determination of the stage of infection in the host.

Investigation of the time of the appearance and disappearance of hypersensitivity which accompany the disease should lead to information which may be utilized to develop a skin test in a manner similar to that used for tuberculosis.

The objectives of this study, therefore, are to investigate the kinetics of the immune response to chlamydial agents, particularly to the psittacosis agent with respect to:

1. The time of appearance and duration of antibodies with different serological activities, especially hemagglutinins, complement fixing and passive cutaneous anaphylaxis antibodies. Also antibodies which are detected by a passive hemagglutination test are being investigated.
2. The appearance and duration of antibodies with

infection neutralization activity.

3. The behaviour of cellular mediated immunity in infected and in inoculated animals at various times after infection and the possibility of utilizing such information for the identification for the presence of active or latent infection.

4. To study the presence of antibodies that contribute to an immediate type allergic reactions using the passive cutaneous anaphylaxis technique, thus contributing to the knowledge of the role of immediate and delayed hypersensitivity reaction in the pathogenesis of chlamydial infections.

LITERATURE REVIEW

Little is known about immunity to psittacosis in man, as compared to that of birds and mammals. Several serological techniques have been devised to study the response of humans and animals to the psittacosis lymphogranuloma venereum-trachoma group. Of these techniques, complement fixation (CF) has been most utilized.

The use of the CF test has shown that the serum of many laboratory workers who were exposed to the agent through working with animal carriers, contained antibodies in significant titers, although these workers did not pass through any suggestive illness.

The titres that result using CF depended on the type of antigen used, whether it was a species-specific or group specific. Cross reactivity is considered to be present among the entire group of Chlamydia. Grayston et al. (1960), have shown that the group antigen from trachoma reacted in high titer with the sera from patients with lymphogranuloma venereum and psittacosis and reacted in low titer or not at all with sera from patients with trachoma.

According to Meyer and Eddie (1962), whenever infected birds are handled for six months to one year, nearly 80% of the breeders, aviary owners, dealers and pet shop owners or employees had CF titers of 1:8 or higher. Since reliable

histories were not always obtained from these workers, only a small percentage were reported as an influenza-like illness.

Smadel et al. (1943) noted that owners of pigeon lofts and squab farms, many with no history of previous illness, had CF titers to the chlamydial agent. They also showed high incidence of clinical psittacosis among pigeon fanciers and their friends.

Kittel (1955) examined the sera of 60 personnel of a duck fattening farm in North Germany after the recognition of two infections, one of which was fatal. He reported that nearly half of them showed CF titers up to 1:3200.

The appearance of CF titers in people who show clinical manifestations of the disease as well as in others that share no clinical manifestations opened the field to the controversy whether antibodies that appear in CF tests were protective or not. There is also the question of the duration of these CF titers.

Irons et al. (1955), in a study of an outbreak of psittacosis in Texas, have shown that of the 22 workers that were infected in 1948, eight were again exposed as pickers and eviscerators in 1951. Of these, five escaped infection, but three suffered reinfection. Nine others, who were exposed in 1948 and escaped infection, became infected in 1951-1952. The attack rate in 1948 was 30 percent; while

in 1951 it was 43.5 percent. In the first outbreak in Texas, CF titers were found in persons who were not ill evidencing exposure without clinical disease. Since the CF test detects antibodies to the whole psittacosis lymphogranuloma venereum group (LGV), lymphogranuloma venereum may not be ruled out. In the second and third outbreaks, CF titers of 12 of 37 workers who were not ill ranged from 1:12 to 1:92. Of these four had psittacosis in 1948. Since in several instances workers who had previously shown titers became ill later, the reliability of the presence of CF titers as a confirmation of immunity, was open to question.

In a screening survey among 309 poultry workers in Wisconsin, Graber and Pomeroy (1958) confirmed the diagnosis of psittacosis in nine inapparent infections and in 10 persons who had recovered but had relapses. Observations were continued in 1959, in which it was reported that human infections did not appear in 1957, but in 1958 two apparent and 11 inapparent infections were discovered among 207 workers. The percentage of persons reacting in the CF test in the workers who had the disease in 1956 or had subclinical infection increased from 16.1 to 29 percent. The CF titers in infected workers however, which ranged from 1:8 to 1:128 maintained their levels for about 32 months. The persistence of antibodies in high titers for 32 months in persons, most of whom were treated thoroughly by antibiotics,

suggests that the antibody production was being stimulated by repeated exposure. They concluded that the persistance of the antibody titer for a long period of time due to reinfection does not necessarily preclude the idea of latency of the infection, nor does it preclude the idea of relapses. Meyer and Eddie (1951) described the case of the 36 year old biochemist who recovered from severe psittacosis and had a CF titer of 1:256 and shed the virus in the sputum for 10 years.

In a later study Meyer and Eddie (1962) reported the case of a woman who had contracted psittacosis during one of the outbreaks in a Texas poultry plant infection and who was treated with small doses of tetracyclines and experienced repeated relapses. During one attack, on the 97th day after the initial illness a virulent bedsonia identical to the turkey isolate was isolated from her blood showing the latency of the agent.

Meyer and Eddie (1962) observed that high antibody levels in parrots and parakeets have consistently been associated with viral latency in spleen or other tissues. These birds have exhibited a relatively high resistance to reinfection, but when they were exposed to some nutritional or other environmental stress the infection would occasionally cause a relapse.

According to Lippellt and Brand (1955), systemic

serologic examination of psittacosis patients showed that in some cases the CF antibodies have persisted for months, even years. For example, about 10 percent of patients convalescent from psittacosis had titers with no tendency to drop from the original high levels within a period of 150 days. Davis (1948) described a survey, where the titer of one serum remained at 1:128 for close to two years. Matthiesen and Volkert (1956) observed that high titers dropped to 1:60 within 15 to 20 months, and then followed a slow steady decrease for 35 months until they disappeared. They assumed that the titers were being stimulated by a latent virus. Further support came from the occasional relapse.

According to Meyer and Eddie (1962) resistance to reinfection does not fully correlate to presence of latent virus. They noted that relatively active immunity was in no way related to latency in avian and mammalian species by clinical and subclinical infection with live virulent or avirulent virus.

Similar conclusions were reported for the immunity to the lymphogranuloma venereum agent. Since this agent belongs to the chlamydial group of agents it is expected to behave in a similar manner as the psittacosis agent. The clinical manifestations of the disease however is distinctly different from that of psittacosis.

According to Meyer and Eddie (1962) many virologists and clinicians state that the CF titers of 1:16 to 1:32 have persisted for decades in untreated patients. With some exceptions, they suggest, that the level of the antibody titer was positively correlated to the duration of the disease. Greaves et al. (1957) described one case of an active infection in which the CF titer persisted at 1:40 for up to 20 years.

The question whether the lymphogranuloma venereum agent confers immunity to the host was studied by Wassen, (1935) and by Caminopetros (1939). Both studies used LGV virus which was heat inactivated for 1 hour at 60°C, and introduced parenterally into man, with the objective of examining its effect in producing resistance to infection with a highly virulent virus. Wassen (1935) injected LGV virus antigen prepared in mouse brain suspension into two groups of subjects at three day intervals. The number of injections received by the two groups were 5 and 10. No local reaction or positive Frei test were observed. Intracutaneous injection of highly virulent material in the thigh on the 14th and 26th day however produced typical clinical LGV.

Similarly, Caminopetros (1939), upon weekly inoculation of four subjects subcutaneously and intravenously with heat-killed antigen prepared from bubos, obtained similar results.

The subjects received injections for 3, 7, 9 or 18 months. In all cases, the Frei tests were negative and subsequent infection of the subjects with live virus by the preputial route and on the right forearm produced typical clinical symptoms. Due to the probable denaturation of the virus by heat treatment, no reliable conclusions were drawn from these two studies.

More work on the immunity and the chlamydial agent has been done in animals than in man, and considerable differences have been shown between the two species. Infection in animals seem to confer more protection than that produced in man. Since the main concern in this research is to relate the immune response in experimental animals to natural infections in man, the discussion related to animals is briefly presented.

Bedson and Western (1930), Rivers et al. (1930), River and Berry (1931) and River and Schwentker (1934) working in parakeets, parrots, rabbits and mice indicated that these birds and animals, after recovery from an experimental injection had a heightened resistance to reinfection after one month.

Rudd and Burnett (1941) and Wagner et al. (1949) inoculated mice with one or two sublethal inoculations of living bedsonias by the subcutaneous route. This resulted in the production of a high degree of protection against

infection by the intra-abdominal route, a moderate or weak protection against infections by the intracerebral route, and no protection against infection by the respiratory route.

Smadel et al. (1943) however, suggested that mice which survived immunization with psittacosis virus were resistant to intracranial inoculation of 10 to 1000 MLD_{50} with the homologous or heterologous strains.

Meyer and Eddie (1933) have shown a high degree of correlation between the immunity states in mice and parakeets, and the persistance of the virus in the spleen and liver. This immunity persisted against massive superinfection.

Meyer and Eddie (1962) however, showed that the infection immunity has occasionally failed in mice when the challenge has exceeded 100,000 MLD_{50} by the abdominal or 100 MLD_{50} by the intracranial route. They also suggested that some living avirulent bedsonia, which do not produce a carrier state, on subcutaneous inoculation, have induced considerable resistance against homologous and even heterologous bedsonias. The antibody response has been vigorous when immunization was repeated. These observations together with the evidence that mice immunized with psittacosis virus inactivated with formalin, but not with heat, induced a partial immunity, still continue to be the subject of experimentation.

Yanamura and Meyer (1942) challenged some vaccinated

mice with intraperitoneal doses of 10 to 100 MLD_{50} of virus. These mice passed through latent infections and became persistent carriers for up to 425 days. Furthermore, Wagner et al. (1946) added that host survival depended on the antigenic mass injected as well as on the procedure of inoculation. Vaccines extremely rich in viral elements administered in repeated properly spaced periods of inoculations or incorporated in oil adjuvants, immunized 75 percent of the mice against 10,000 to 1,000,000 MLD_{50} given intraperitoneally.

Meyer and Eddie (1962) indicated that reaction of mice inoculated abdominally with inactivated avian and mammalian bedsonias was always relative. Individual mice have been seen to be so resistant that they cleared their tissues from challenge infections with 100 to 500 MLD_{50} within few days, while those challenged with higher doses acquired a chronic infection. Fatal results were reached when the challenge dose exceeded 10^6 MLD_{50}. The resistance has been invariably weak against respiratory challenge and even weaker against intracranial challenge. In another experiment five weekly intranasal instillations of formalin-inactivated bedsonias elementary bodies in mice conferred resistance against respiratory infection with 100 MLD_{50} to at least half of the animals. Some clinically well mice sacrificed on the 10th day had typical focal pulmonary lesions; by the 40th day there were splenic carriers with chronic pneumonia.

They also showed that direct immunization of the respiratory tract was not sufficiently effective to prevent infection by the same route, but might reduce multiplication of the organism below the level that would cause death.

Bedson (1938) stimulated complement fixing and protective neutralizing antibodies with formalin inactivated vaccine in some mice. Although the peripheral antibodies gradually declined in some mice, the immunity lasted for three to six months after the last inoculation.

The state of immunity in the guinea pig seem to differ from that of the mouse. Meyer and Eddie (1962) reported that in guinea pigs, the carrier state does not necessarily substantiate immunity. It was found that animals, sacrificed at 40 days after the time of challenge with bedsonia had completely recovered from the initial infection and had sterilized their tissue by the 40th day. It was also found that 10-20 animals, sacrificed 40 days after an infection with bedsonia from various sources had completely recovered from the infection and had sterilized their tissue. In addition a significant proportion of the animals challenged 40 days after the injection survived. They suggested that the guinea pig provides an excellent tool for the evaluation of the antigenicity of inactivated bedsonia vaccine, due to their powerful antibody response, particularly with vaccines in adjuvant.

Although the CF technique has been the most widely serological technique utilized to detect the presence of the chlamydial agent, other techniques such as the passive cutaneous anaphylaxis test, the neutralization of infectivity test, and the hemagglutination test (HA) have been also used. The passive cutaneous anaphylaxis (PCA) is an exaggerated cutaneous reaction which occurs in a normal host as a result of the injection of the serum of a previously sensitized host with the specific antigen, Arey *et al.* (1957). Although no attempt has been made to elicit PCA to the chlamydial group of agents, Ormsbee *et al.* (1968) reported a similar study on *Rickettsia prowazeki* agent. Since the chlamydial group of agents belong to the order of Rickettsiales this study might be applicable to chlamydial agents. The authors studied the effect of particular fraction of gamma-globulin extracted from guinea pigs following exposure to the antigens of *C. burnetti* or *R. prowazeki* in the elicitation of the PCA, and the production of protection against disease. Using DEAE-cellulose columns to fractionate the sera, they demonstrated that the concentration of the PCA activity was mainly around the second (fast 7s gamma-globulin) and the third protein peaks. Although a specific PCA inhibitor was found in the first peak (slow 7s gamma-globulin), no PCA activity could be detected in that peak.

The study of the PCA phenomenon in chlamydial infections might shed some light on histopathological conditions which

accompany the disease and which at the time have no explanation.

The infectivity neutralization test is a specific technique whereby an agent which induces the formation of a specific neutralizing antibody, when mixed with the antibody, will render the mixture non-infectious. Studies on the use of neutralization of infectivity in mice have been reported by Hilleman (1945) and St. John and Gordon (1947); in chick embryos by Moulder et al. (1958), and Jenkins et al. (1961); and in cell culture by Reeves and Graham (1962) and Blyth et al. (1962).

Hilleman (1945) prepared specific neutralizing antisera against meningopneumonitis, lymphogranuloma venereum and mouse pneumonitis agents by intraperitoneal inoculation of chicken. The antiserum prepared against mouse pneumonitis agents was shown to be capable of agglutinating the elementary bodies of the same agent in the test tube and by a rapid slide agglutination technique. Furthermore, a definite protective effect of the mouse penumonitis antiserum on the experimental infection in mice could be demonstrated when the antiserum was given either before or after inoculation of the agents. St. John and Gordon (1947) prepared a neutralizing antiserum in roosters. The infectivity test was carried out by intranasal inoculation into mice. By cross reacting the neutralizing antisera against different

chlamydial agents they were able to utilize the infectivity-neutralizing antigen to differentiate between different species of chlamydial agents, eg. feline pneumonitis antiserum neutralized its homologous agent but did not protect against meningopneumonitis, mouse pneumonitis or lymphogranuloma venereum agents. This was the first test which used an immunogen to differentiate between different chlamydial species.

Moulder et al. (1958) prepared a neutralizing antiserum against feline pneumonitis agent and applied the serum neutralization test. The agents and the antiserum were incubated four hours at 25°C and then inoculated into the yolk sacs of groups of 6 days old chick embryos. Neutralization of infectivity was manifested by increase in the survival of embryos inoculated with mixtures of the agents and the neutralizing serum. The neutralizing titers were unchanged by inactivation of the serum at 56°C for 30 minutes, indicating that the neutralizing antibody was heat stable. Jenkins et al. (1961) managed to show in their cell wall preparation an infectivity-neutralizing antigen by reacting the meningopneumonitis and feline pneumonitis agents with fowl antiserum and tested the infectivity of the neutralized agents in chick embryos. The antigen was shown to be species-specific because the specificity of the absorption is shown by the failure of feline pneumonitis

agents to absorb neutralizing antibody for meningopneumonitis agents.

Reeves and Graham (1962) demonstrated the neutralization of infectivity in a test using TRIC agents in HeLa cell cultures with specific antiserum from immunized rabbits. The index of neutralization of infectivity was indicated by the reduction in number of plaques formed by the agent. Similarly Blyth et al. (1962) produced sera of a very high concentration of neutralizing antibody with Inclusion conjunctivitis agents in rabbits by seven subcutaneous inoculations of oil-emulsified, living antigen prepared from yolk sac membranes. The infectivity-neutralization tests were made in HeLa cell cultures by their cell-inclusion counting method. In addition to confirming the presence of an infectivity-neutralization antigen in the chlamydial agents, Blyth et al. (1962) also found that the production of the group-specific CF antibody, though present, failed to parallel the production of the neutralizing antibody. This provided evidence that the CF antibody was not the neutralizing antibody.

The Hemagglutination Reaction (HA), is a test by which a certain agent agglutinates red blood cells. Hilleman et al. (1951) using the indirect CF hemagglutination and conglutinating complement absorption tests for viruses of the psittacosis-lymphogranuloma venereum group, demonstrated

that the hemagglutinins of the meningopneumonitis were similar to those of the vaccinia-variola-ectromelia group 19 of agents with respect to the separability of most of the agglutinating substances, from the virus.

The hemagglutination reaction could be demonstrated with different viruses, bacterial antigen and chemical agents. For this reason a hemagglutination reaction for Chlamydia would be considered non-specific unless it is coupled by an inhibition of the reaction with specific antisera to Chlamydia.

Using the hemagglutination inhibition (HI) test, Gogolak and Ross (1955) inhibited the hemagglutination of five units of the hemagglutinins of the 6BC agent of psittacosis by a 1:5120 dilution of the anti 6BC serum, and 10 units of the hemagglutinins by a 1:1280 dilution of the serum. They also demonstrated cross reactivity of the hemagglutination reaction within the psittacosis virus, using pisttacosis 6BC, and feline pneumonitis hemagglutinin sensitized cells, and were able to inhibit the hemagglutination reaction by identical dilutions of anti 6BC sera, thus confirming the group specificity of the hemagglutination inhibition reaction within the psittacosis-lymphogranuloma venereum virus group. They also demonstrated that the titers obtained in the HI test, with sera from roosters immunized with viable psittacosis virus, were greater than

those obtained with CF and agglutination techniques.

Benedict and O'Brien (1958), on the other hand, using passive hemagglutination reaction for psittacosis, demonstrated that the CF and hemagglutination reactions measured different antibodies in sera from experimental animals. The percentage of titers showing positive results when tested by the CF and HA techniques were 80 and 70, respectively.

Vedros (1967) using the indirect hemagglutination (IHA) technique in an attempt to produce a standard serologic method for demonstrating immunologic differences within the genus Chlamydia, was able to differentiate species specific antigens of the TRIC strain of trachoma from those of the 6BC strain of psittacosis. This antigen was demonstrated to be heat sensitive at 56°C for one hour and reacted with antibodies which were different from those measured by the CF test.

The Chlamydial agents possess antigens capable of eliciting a specific antibody response detectable by several serological techniques, and immunogens capable of producing protection. It has already been pointed out that protection is not complete and that the effect of the immunogens can be detected by either neutralization of infectivity or by protection against the toxicity of the agents.

Antigens of chlamydial agents can be categorized into

two main categories, the group specific antigens, which are shared by all the chlamydial agents, and the species specific antigens. The group specific antigens have been shown to be heat stable by Bedson (1936) who was able to use boiled psittacosis as a source of antigen in the CF test. This was later clarified and confirmed by the works of Barwell (1952), Benedict and O'Brien (1956) and Ross and Gogolak (1957). Each of the species specific antigen is possessed by one species only. Their preparation or detection is only possible after the group specific antigen is removed by absorption with the group specific antibody. The species specific antigen have been shown to be heat labile, Benedict and O'Brien (1956).

Both the group specific and species specific antigens can be detected by CF tests, fluorescent antibody technique, immunodiffusion technique, hemagglutination, radioisotope precipitation test and infectivity neutralization tests. Other antigens present with a group or specific specificity include the toxic substances and the skin testing antigens.

The relationships between different antigens and antibodies have been studied by several workers. Benedict and O'Brien (1958), using the passive hemagglutination (PHA) test were able to demonstrate that the CF antigen and the hemagglutinating (HA) antigen tests utilized different antibodies. In addition the hemagglutination produced by

PHA technique was inhibited by the antibody specific for the HA, while the CF activity remained unchanged.

Sayed et al. (1971) using the in vitro inhibition of macrophage migration technique correlated cellular immunity and skin-test antigens. They obtained a positive correlation between the skin test reaction and the cellular immunity reaction using the same antigen.

Collins and Barron (1970) found that sera with detectable CF antibody level normally gave positive reaction in gel diffusion studies, suggesting the CF antigens and those detectable by immunodiffusion techniques would probably be present in the same macromolecule.

Gerloff and Watson (1967) from the results of a study of the CF antibody and the radioisotope precipitating antibodies using sera from patients with different chlamydial infections, concluded that the two tests detected different antibodies. Significant differences in the titer between the sera obtained by the two tests from the same patients were demonstrated.

The relationships between infectivity, CF and fluorescent antibody technique (FA) antigens were studied by Ross and Borman (1962). They compared the development of infective particles, the FA and the CF antigens in mouse lung tissue cultures infected by mouse pneumonitis agents. During the first 24 hours, there was a decrease in infectivity and no

CF antigen was detected.

Attempts to produce an effective vaccine against the trachoma agent (TRIC) were initiated after Tang (1957) who was able to propagate the trachoma agent. Most studies since, were concerned with this agent. The production of vaccines against the other chlamydial agents was neglected.

Collier et al. (1958) inoculated human subjects with the G_1 strain isolated from a trachomatous patient in Gambia. The initial CF titers on the sera obtained from those subjects prior to inoculation were 1:64 to the G_1 strain and 1:32 to the Tang strain and Te_{55}, and 1:64 to the psittacosis antigen. The titers against these three antigens rose to 1:128 on the 42nd day after inoculation with the G_1 strain. It was concluded that the subjects had the possibility of past infection with another virus of the psittacosis-lymphogranuloma group, although no history suggesting or supporting that was reported, and that they were not protected against the experimental infection. The small rise in CF antibody during the course of experimental infection is in accord with what is known to occur in the natural disease.

Bernkopf et al. (1960) used an agglutination technique to test for the presence of antibodies to the trachoma agent propagated in yolk sac. The sera obtained from immunized rabbits showed agglutinating titers of 1:200 to 1:400 which when examined by the CF test gave a lower percentage of

positive results.

Julianelle (1939) used a virus suspension obtained by scraping the conjuctiva of clinically active patients to inoculate Macaccus rhesus monkeys. One eye was inoculated by swabbing the conjuctiva of the everted lid while the conjuctiva of the other eye was multiply pricked with a syringe containing the agent. The monkey was then sub-conjunctivally injected with 0.2 cc of the same suspension. It was concluded that although clinical observation revealed very little evidence for immunity against trachoma, increased resistance to experimental trachoma in monkeys following the recovery of infection was demonstrated. He also found that the serum of patients with active infections of varying duration excreted no neutralizing or protective effect on the virus of trachoma and that the virus of trachoma was an impotent and ineffectual antigen.

Collier (1966) reviewed studies on vaccines (Table 1a). Most of the studies on trachoma vaccine demonstrated that the CF titers detected as a result of vaccination had no correlation with protection against the disease.

Most of the work presented in this review has dealt with the humoral aspect of immunity. A review of the studies on the cellular aspect of immunity that is produced by the psittacosis-lymphogranuloma venereum group of agent is presented in the following pages. The interaction between the agent and the host in chlamydial infections has not been clearly evaluated.

TABLE 1a

Studies on Trachoma Vaccines.

	Research Group	Centre	Type of Vaccine
1)	Bell, Theobald, Snyder, and Murray	Harvard School of Public Health	ether treated yolk sac suspension
2)	Grayston, Wang, and Woolridge	United States Naval Medical Research Unit No. 2 in Taiwan	purified formal inactivated agents emulsified in mineral oil adjuvant
3)	Dansen and Jawetz	Francis I, Proctor Foundation, U.S.A.	crude live yolk sac suspension vaccine
4)	Medical Research	Trachoma Research Unit in London, Fajora and Gambia	a) formal inactivated yolk sac suspension b) heat inactivated yolk sac suspension

Wyman et al. (1970) suggested that latency or chronic and subclinical infection where the organism seems to remain inside the cell for long periods of time, was the cause of high and relatively long lasting antibody levels for psittacosis among the Eskimo population in Northern Canada.

Bietti et al. (1967) studied the correlation between the appearance of dermal reactions, associated with the psittacosis lymphogranuloma venereum (PLTV) infections, with the state of latency that is produced in these infections. The persistance of the PLTV in the tissues of the host in a latent form tends to elicit a cutaneous hypersensitivity in man and animals. This hypersensitivity can be revealed easily by the injection intradermally of a suitable antigen.

While skin reactions have been detected in many of the agents of the chlamydial group, such as LGV, psittacosis, cat pneumonitis, meningopneumonitis, and mouse pneumonitis, cross reactions between these agents have also been demonstrated. Although cross reactivity has been shown to occur using CF as a serological technique, Kilham (1948) obtained a higher correlation for cross reactivity using the CF test, than that of the skin test. This difference was explained by the higher sensitivity of the CF test.

In addition Barwell (1952) found that the antigens for the two tests were different and that the CF activity was abolished by periodate treatment, while the skin

reactivity was retained. Benedict and O'Brien (1958) have also shown that there was an increase in the titer tested by passive hemagglutination after skin testing. The production of an anamnestic response after skin testing affected the hemagglutination test.

Bietti et al. (1967), experimenting on some clinical cases of trachoma, were able to produce skin reactions in cases that did not show any clinical infection as well as in those who had a low serum antibody level. In addition, they transferred the hypersensitive state to non-reactive individuals by injecting animals intradermally with leucocytes obtained from the positive donors.

The question whether this hypersensitive state had any relationship to the damaging effect produced by the disease, or to the pathological lesions produced in individuals, such as the hypersensitive state produced in tuberculosis, is still not very clear.

Meyer (1965) suggested that the lesions produced by psittacosis infections were similar to those of tuberculosis. He described the histopathological picture in psittacosis and ornithosis as an invasion of lymphocytes and macrophages into the alveolar spaces. In lymphogranuloma venereum, the inflammatory lesion seem to be an outpouting of plasma cells, and a formation of giant cells by the macrophages.

To relate the hypersensitive state with the true

cellular-mediated immunity reaction, it is important first to put criteria characterizing the delayed hypersensitivity state. Sell and Asofsky (1968) proposed the following criteria:

1) delayed hypersensitivity can be transferred by specifically sensitized cells, not with serum or by a sub-cellular fraction,

2) the time course is more prolonged for the skin reaction, it may last for days or weeks and,

3) the histopathological picture of the reaction site show infiltration of cells, mostly monocytes, at the site of the antigen, with subsequent damage.

David et al. (1964 a, b and c) used an in vitro technique to study the cellular mediated immunity in tuberculosis. This technique was based on the observation that when macrophages are obtained from sensitized animals, and placed in a capillary tube in the presence of culture medium and the sensitizing antigen, the normal migration of these macrophages will be inhibited.

Fauve et al. (1969) modified David's method by making a slide technique for the macrophage spreading inhibition test (MSI) in which a number of macrophages spreading over a glass surface in a hemocytometer is counted.

David et al. (1968) and Fauve and Dekaris (1969) using the macrophage inhibition test (MI) and the MSI,

respectively, studied the relationship between the in vitro tests and the in vivo skin reactions. Both studies showed that subjects with high levels of macrophage inhibition (MI) and MSI, have a more pronounced skin reaction and vise versa.

Studies in our laboratory by Nicks et al. (1970) and Sayed et al. (1971), using the lymphogranuloma venereum agent, with sensitized guinea pigs and mice tested by MI and MSI respectively showed that a cellular mediated hypersensitivity was present in animals sensitized by the agent. It was postulated that similar reactions exist for other chlamydial agents. The dermal reactions of LGV was also shown to be caused by an infiltration of macrophages and lymphocytes. A positive correlation between degree of the dermal reactions and the MIT and the MSI index was demonstrated. Several components were also isolated from the agent using the electrofocusing technique which were reactive with sensitized macrophages, and monocytes. These components contained a high protein nitrogen content but a relatively low amount of polysaccharide.

Sayed and Wilt (1971) studied the changes in serum proteins in humans due to psittacosis infections. They measured the levels of several proteins, α_2-macroglobulin, α_1-antitrypsin, α_1, acid glycoprotein (AGP), IgM, IgG and IgA in the sera of sixteen patients from Manitoba, nine of

which were diagnosed between 1958 and 1968, the seven other patients in 1970. The tested sera were obtained up to 200 days from the onset of clinical signs. Blood samples were also collected after 20, 40, 120 days. The results of this study indicated an elevation or sera IgM of most patients at about 20 days, and no further change during the course of the disease. The IgG level was found to be persistantly low at all times during the disease and significantly lower than the level found in normal healthy persons. The IgA level however was found to increase after 20 days, but it returned to the normal level by the 200th day. The level of α_2-macroglobulin increased at the 20th day and then returned to the normal level by the 200th day, while the level of α_1-antitrypsin showed a decrease at 20 days and returned to normal at 200 days. It was postulated that the chronicity and recurrence of the disease did not affect the normal levels of the antibody response observed in the patients studied. The unusual level was postulated to be due to either a deficiency in the cellular mediated immunity, or to the property of the organisms. The elevated levels of α_2-macroglobulin and α_1-AGP were shown to behave as an acute phase reactant. No explanation was given to the low levels of the α_1-antitrypsin under the experimental conditions.

MATERIALS and METHODS

Agent

Inactivated DD strain of psittacosis was obtained from Markham's Laboratories Chicago, U.S.A. It had been propagated in the yolk sac of embryonating chicken eggs. The agent was not treated chemically but was inactivated by several washings in buffer containing 1:100 phenol. A 1% residual infectivity was thought to be contained within the antigenic preparation. The N_2 content was estimated at about 80 mg/ml by the Lowry technique.

Infective DD strain of psittacosis was also obtained from the same laboratories. This agent was also prepared in the yolk sac and was estimated to have 10^{-3} MLD_{50}/ml of phenol.

Animals

CF_1 and $BALB/C_1$ strains of mice were used. In each experiment a total of 185 mice were divided into 5 groups in the following manner.

1) Group A Fifty female mice of the CF_1 strain were divided into 5 sub-groups, each containing 10 mice.

2) Group B The fifty male mice of the CF_1 strain were divided into 5 sub-groups each containing 10 mice.

3) Group C Fifty mice were also included in this group. They were all females of the $BALB/C_1$ strain, and were divided equally into 5 sub-groups.

4) Group Z Twenty mice, all females of the CF_1 strain were divided equally into 2 sub-groups.

5) Group M Fifteen male mice of the CF_1 strain were divided into 3 equal sub-groups.

All animals were tested and were found negative for a group complement fixation antibody to the psittacosis agent.

A) Injection Procedure

Group A

All the mice in this group were injected intraperitoneally with 0.5 ml of the non-infectious DD strain of psittacosis containing 0.5 mg/ml of N_2. The original preparation of the concentrated agent was found to contain 80 mg/ml of N_2 by the Lowry technique, and in order to obtain the dilution of 0.5 mg/ml glass double distilled water was used. The 0.5 ml of injected material consisted of 0.25 ml of the agent and 0.25 ml of Freund's complete adjuvant.

Group B

The mice in this group were also injected intraperitoneally with 0.5 ml of the same agent, except that dilution of 1 mg/ml was used instead of 0.5 mg/ml. As before, glass double distilled water was used for diluting the agent. The amount injected consisted half and half of the agent and the Freund's complete adjuvant. A booster dose in the same amount of the agent in Freund's Complete Adjuvant was given at 22 days after the initial injection.

Group C

This group was injected by the oral route. Each mouse

received 0.05 ml of the infectious agent containing 10^{-3} MLD_{50}. The mice were partially anaesthesized with ether and the agent was introduced into the nasopharynx using a syringe with a blunt needle.

Group Z

This group was also injected intraperitoneally with 0.5 ml of normal allantoic fluid, and the amount injected into each animal also consisted half and half of the normal allantoic fluid and Freund's complete adjuvant. This group of mice served as controls.

Group M

The mice in this group received 0.05 ml non-infectious DD strain of psittacosis in two foot pads, and contained a mixture of equal volumes of the agent and Freund's complete adjuvant. The N_2 content of the injected material was 2 mg/ml.

B) Estimation of N_2 Content

The Lowry technique (1951) was used with a slight modification as follows:

Solution A - contained 2% of Na_2CO_3 in 0.10 N of NaOH, and was made up to 500 ml with glass double distilled water.

Solution B - 0.5% of $CuSO_4 - 5H_2O$ and 1% of Na tartrate were dissolved in 50 ml of glass double distilled water.

Solution C - contained 50 ml of solution A and 1 ml of solution B

Solution D - Folin reagent was diluted to IN by adding glass double distilled water.

Procedure:

One-fifth of millilitre of the non-infectious DD strain of psittacosis was diluted with distilled water to make the required dilutions. 5 ml of reagent was added and mixed, this was left at room temperature for 10 minutes. Then 0.5 ml of reagent D was added and mixed immediately. The optical density was read after 30 minutes at 750 using a Unicam SP500 single beam spectrophotometer, and the results were obtained from a standard curve made from standardizing bovine serum albumin (BSA) under the same experimental conditions.

C) Scheme of Collection of Blood from Mice

A system was established to bleed mice in such a way that no single mouse would be bled more than once per two weeks. Up to 21 days blood was collected from each sub-group A and B at an interval of 3 days. At the end of 21 days blood was collected after one week, and then at an interval of 5 days up to the 58th day. Group B mice were reinjected on the 21st day with the same dose as the first time.

Group C For first 8 days, the mice were bled every 2 days, and then on the 13th and 15th day and on 22nd day all the animals were bled to death. The number of deaths at each interval was also recorded.

Method of Bleeding and Serum Collection

The mice to be bled were placed in a small cage and a 150 watt lamp was lit over the cage in order to warm the animals which facilitates bleeding. After about 10 minutes under the heat of the lamp, the mouse was introduced into a hollow 50 ml syringe with the tail hanging down. The tail vein was then cut with a blade, and the blood was collected drop by drop into a glass test tube.

The blood from mice of the same subgroup collected on the same day was pooled, left in a slanting position in the test tube at room temperature for 2-3 hours, and was then refrigerated overnight. The blood was then centrifuged at 1000 r.p.m. for 10 minutes and the serum was separated and frozen until further use.

D) Serological Techniques Employed for Analysis of Serum Antibodies

1) Complement Fixation (CF) Test:

The microtechnique of Takatsy (1950) was followed with slight modification.

TABLE 1

Bleeding Schedule for Groups A and B

Subgroups	Day of Injection	Days after Injection of Mice													
		3	6	9	12	15	18	21	24	33	38	43	48	53	58
1	0	x					x					x			
2			x					x					x		
3				x					x					x	
4					x					x					x
5						x					x				

TABLE 2

Bleeding Schedule for Group C

Subgroup	Day of Oral Infection	Days of Bleeding						
		2nd	4th	6th	8th	13th	15th	22nd
1	0							x
2					x			x
3				x				x
4			x				x	x
5		x				x		x

TABLE 3

Number of Deaths Produced

	Days of Death							
	0	2nd	4th	6th	8th	13th	15th	22nd
No. of Deaths	2	8	2	2	1	2	1	32

Reagents

a) Diluent - Veronal buffer metal solution (VBM)

Solution A - 5.75 gm of diethyl barbituric acid were dissolved in 500 ml of hot distilled water.

Solution B - 85.0 gm of NaCl and 3.75 gm of Na-5, 5 diethyl barbiturate were dissolved in 1500 ml of distilled water.

Solution A and B were added together to give 2000 ml of solution.

1.015 gm of $MgCl_2.6H_2O$ and 0.220 gm of $CaCl_2.2H_2O$ were dissolved in 2 liters of distilled water.

This constituted the stock solution, which was put in glass stoppered bottles and placed in the cold room at 4°C.

Before use, this stock solution of V.B.M. was diluted 1:4 with 5% glucose solution. This buffer was used for all the dilutions required in the CF test.

b) Sensitized Cell Suspension

i) Cells

Citrated sheep red blood cells were collected in equal volume of Alsever's solution obtained from the National Biological Company, Winnipeg, Manitoba. These cells were centrifuged at 1000 r.p.m. for 10 minutes at 4°C, and the serum was removed and discarded. The cells were then washed, and spun at 1000 r.p.m. for 10 minutes at 4°C 3 times with V.B.M. diluted 1:4 with 5% glucose solution. A 10%

suspension of the cells was then prepared with the diluent and left at 4°C. This suspension was used for about 1 week.

ii) Hemolysin

A 1:100 stock solution of hemolysin was diluted in one fold dilutions up to 64 thousand and was titrated with 0.5% cell suspension in the microplastic plates. A 1:30 dilution of complement (C') was added to the suspension, and was incubated at 37°C for 30 minutes, and the results were then read. The titer obtained represented the highest dilution of hemolysin which showed complete hemolysis. This titer represented one unit of hemolysin. The following 3 controls were used:

1) containing sheep red blood cells, the diluent and no C' or hemolysin
2) containing sheep red blood cells, the diluent, and the hemolysin but no C'
3) containing sheep red blood cells, the diluent, and the C' but no hemolysin.

To sensitize the cells with the proper dilution of hemolysin, 0.5% suspension of cells were added to equal amount of the proper dilution of hemolysin, mixed, and allowed to stand for at least 10 minutes at room temperature prior to use.

iii) Complement

The titration of C' was performed by making a serial dilution of C' with diluent, and the sensitized sheep

red blood cells were then added to it in a plastic plate. This was then incubated at 37°C for 30 minutes and the results were read. The highest dilution of C' that showed complete lysis of the cells constituted one unit. 2 units of the C' were used in all the CF tests.

iv) Antigen

A 1:16 dilution of psittacosis human-pneumonitis diagnostic antigen obtained from Markham Laboratories in Chicago was used for all the CF tests.

v) Serum

The serum obtained from mice was heated at 57°C for 30 minutes in order to inactivate the C' present in that serum.

Procedure for Complement Fixation Test:

Two-fold dilutions of inactivated serum were prepared in U-shaped cups of a plastic plate with 0.025 ml loops. Then a 1:16 dilution of the commercially prepared antigen was added in a volume of 0.025 ml with a dropping pipette. Two units of C' were then added in a volume of 0.025 ml. The plates were sealed with a tape and incubated at 37°C for 30 minutes. After incubation, 0.025 ml of 0.5% suspension of sensitized cells were added to each cup with a dropping pipette. The plates were shaken and returned to the 37°C incubator for 30 minutes. The results were then read.

Controls:

1) Serum controls: A serial dilution of the serum to be analyzed was used with C' - and sensitized cells but without the antigen to detect any anti-complementary effect in the serum.

2) Antigen controls: Two wells were used which contained the Ag the sensitized cells and the C' but no serum.

3) Sensitized cell controls: Two wells contained the sensitized cells, and the C' but no serum or antigen.

4) Cell control: Two wells contained sensitized cells and buffer only.

2) Hemagglutination Inhibition Test (HI)

The technique used by Hilleman (1951) was followed here with slight modification.

Reagents

a) Diluent

McIlvaine buffer of pH 7.0 and ionic strength 1.0 M was used after Elving et al. (1956). To prepare this buffer 58.9 gm of $Na_2HPO_4.12H_2O$ and 3.70 gm of $H_3C_6H_5O_7.H_2O$ were dissolved in 1 litre of double distilled water. To bring the ionic strength of the buffer to 1.0 M, 42.7 gm of KCl were added per litre of the solution. The solution was

kept at 4°C and served as a stock solution. The diluent used for all the preparations and in the HI test, consisted of 1 part of the buffer and 4 parts of physiological saline.

b) Erythrocytes

White leghorn citrated red blood cells were collected in an equal amount of Alsevar's solution were obtained from the Department of Veterinary Science, The University of Manitoba, Winnipeg, Manitoba.

The cell suspension was spun at 1000 r.p.m. for 10 minutes at 4°C and the serum was discarded. The cells were then washed and spun down at 1000 r.p.m. 3 times with McIlvaine buffer and physiological saline 1:4 mixture. 10% suspension of cells were then prepared and were left at 4°C and were used for approximately two weeks.

c) Antigen

The antigen was prepared from psittacosis 6BC grown in L-cells maintained in Eagles 199 media. The infected cells were collected, frozen and thawed 3 times in dry ice to break the cells, and release the agent. Hemagglutinating antigen was then obtained by the method of Barron et al. (1965) and Barron and Riera (1969) with some modifications.

Titration of the Antigen

A serial two fold dilution of the antigen was prepared in McIlvaine buffer and saline 1:4 using the microtechnique.

This entailed placing 0.025 ml of the buffer in several cups of a U-shaped plastic plate. 0.025 ml of the antigen was then added to the first cup, mixed with the buffer and 0.025 ml were transferred from the first to the second cup and so on up to the desired dilution. 0.025 ml of 0.5% suspension of chicken RBC was then added to each cup. The plates were covered and left at room temperature for 1 hour, and then results were read. The hemagglutinating titer was the highest dilution of the antigen that hemagglutinated the cells.

d) The Serum

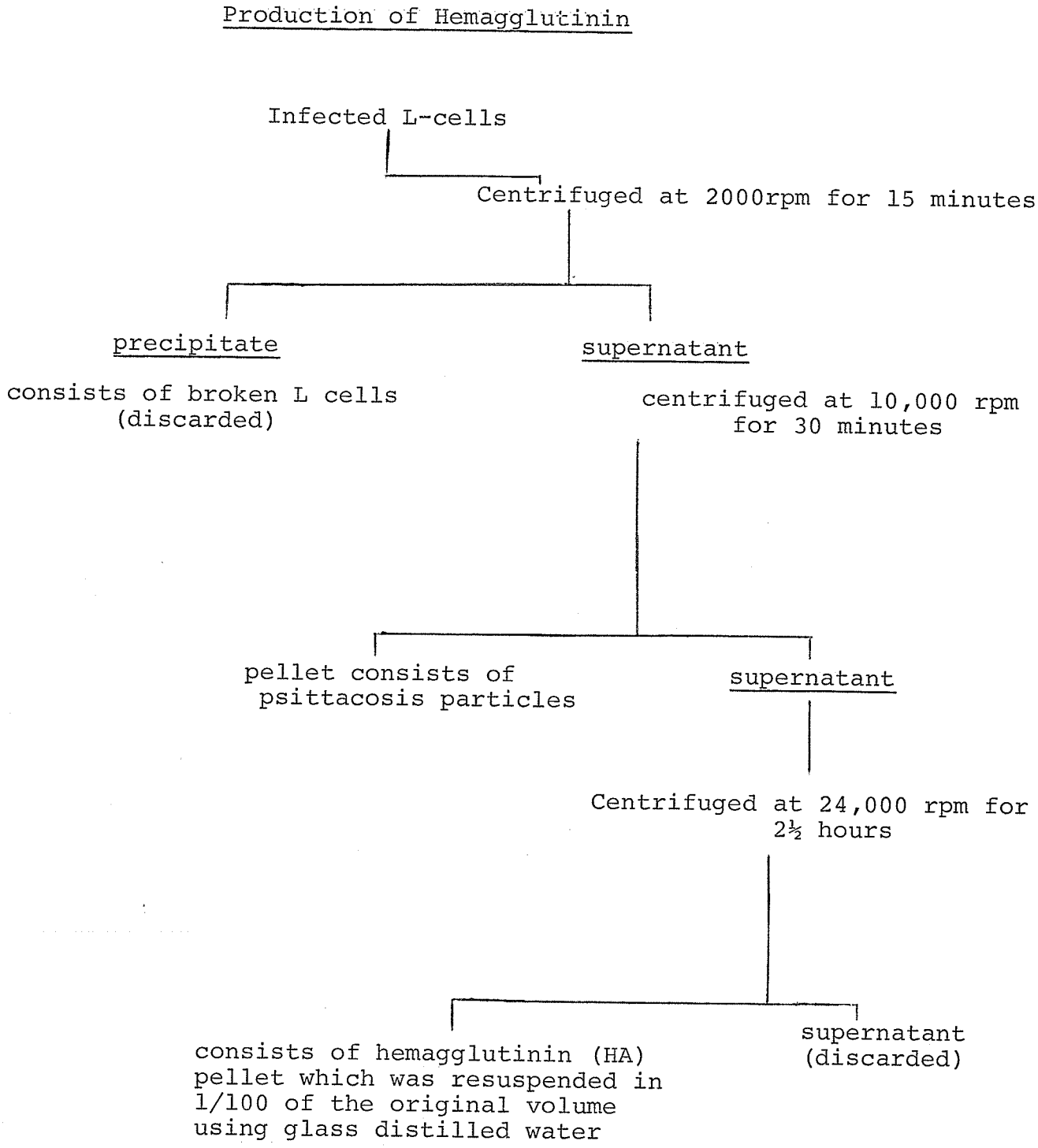
The serum used was the one obtained from mice. Sera were heated at 56°C for 30 minutes before use. Some sera were absorbed with chicken erythrocytes at 4°C for 20 minutes.

e) The Hemagglutination Inhibition Test

Two-fold serum dilutions were made using 0.025 ml loop in U-shaped plates. Four units of antigen were added to each cup in a volume of 0.025 ml. Then 0.025 ml of 0.5% suspension of cells was added to each well, and the plates were incubated at room temperature for about 1 hour. The serum titer was then read as the highest dilution that inhibited hemagglutination.

Controls

1) Serum control: One well containing the serum and


Production of Hemagglutinin
Infected L-cells
Centrifuged at 2000rpm for 15 minutes
precipitate
consists of broken L cells (discarded)
supernatant
centrifuged at 10,000 rpm for 30 minutes
pellet consists of psittacosis particles
supernatant
Centrifuged at 24,000 rpm for 2½ hours
consists of hemagglutinin (HA) pellet which was resuspended in 1/100 of the original volume using glass distilled water
supernatant (discarded)

the cells only.

2) Antigen control: One well containing the antigen, and the cells only, without the serum,

3) Cell control: One well containing only a suspension of the cells in buffer.

3) Macrophage Spreading Inhibition Technique (MSI)

The procedure followed was that of Fauve et al. (1968) and Fauve et al. (1969) with certain modification.

Mice to be used for this test were killed by ether, and were immediately injected intraperitoneally with 5.0 ml of Eagles 199 medium, in order to enhance the survival of the macrophages. The abdomen was then massaged, to obtain the maximum number of macrophages.

The macrophages were then obtained from the peritoneal cavity with the aid of a 5 ml syringe and 21 guage needle, and placed in clean glass tubes.

The test tubes were then spun at 900 r.p.m. for 5 minutes to sediment the macrophages. The supernatant was discarded and the macrophages were resuspended in 0.6 ml of fresh Eagles 199 medium. The suspension was then divided equally between two tubes. To one tube 0.1 ml or a drop of the specific antigen was added which was used to inject the animals. The tubes were then incubated at 37°C for 30 minutes. A drop from each sample, with and without the antigen was

then placed on a hemocytometer and was incubated at 37°C for 30 minutes. After incubation the hemocytometers were read under a phase contrast microscope, and the number of macrophages spreading in the absence of the antigen was compared with those spreading in presence of the antigen.

The MSI index was then calculated as follows:

Without Antigen	With Antigen
$\frac{\text{No spreading}}{\text{Total number of cells present}} = x$	$\frac{\text{No spreading}}{\text{Total number of cells present}} = y$

$$\text{MSI Index} = \frac{y}{x}$$

The spreading macrophages were distinguished from non-spreading ones by the shape of the macrophage. The spreading macrophages show wrinkled edges, while the non-spreading ones are usually rounded.

4) Fractionation of Serum

I) Sucrose Density Gradient Ultracentrifugation

Fractionation was performed on the mice sera from groups A, B, C and Z, by the method of Kim (1964). Each cellulose tube (Beckman ½ x 2" in size and 5 ml capacity) to be used for fractionation contained 2.3 ml of 30% and 2.3 ml of 10% sucrose in VBM. The 0.5 ml of serum sample was layered on top of the sucrose gradient. Prior to layering of the serum sample, the sucrose gradient was left at room temperature for about 4 hours, to achieve equilibrium.

The sucrose gradient with the serum sample was then ultra-centrifuged in a precooled Spinco SW39 rotor at 39,000 r.p.m. for 16 hours. At the end of the run, the sample was collected from the top in 0.5 ml fractions using 50% sucrose. Each sample was then dialysed in narrow dialysis bags for 24 hours in physiological saline, and in VBM for further 24 hours. Each sample was then collected from the dialysis bags and was frozen until further use.

II) Immunoelectrophoresis

Reagents:

a) Veronal Buffer (VB)

Stock A = 41.2 gm of sodium barbital was dissolved in 1000 ml of glass-distilled water. To this was added 1 gm of sodium azide.

Stock B = 2 ml of 10 M HCl mixed with 98 ml of glass-distilled water to obtain a 0.2 M solution. 750 ml of stock A was mixed with 90 ml of 0.2 M of HCl and 2,160 ml of glass-distilled water to obtain 3 liters of the buffer, and its pH was adjusted to 8.6.

b) Agar

Half a gram of Noble Agar was dissolved in 10 ml of VB and 40 ml of glass-distilled water and 0.125 ml of sterile metal solution (1.015 gm of $MgCl_2.6H_2O$ and 0.22 gm of

$CaCl_2 \cdot 2H_2O$ dissolved in 2 liters of distilled water). The pH was adjusted to 8.6 and the solution was warmed to enhance dissolution.

c) Amido Schwartz Stain

One-twentieth of a gram of Amido Schwartz 10 B dye was dissolved in 20 ml of acetone-free absolute methanol, and then 20 ml of glass-distilled water and 4 ml of glacial acetic acid were added.

d) Ponceau Stain

500 mg of the stain was dissolved in 100 ml of 5% trichloracetic acid.

e) Preparation of Slides

Several glass slides were first washed with alcohol and dried. They were coated with 0.5 ml of agar and were allowed to dry at room temperature. Then 2.5 ml of the agar were added to each slide, and the slides were allowed to solidify in a moist chamber at 4°C.

A central trough 2 mm in diameter was cut, and immediately on either side of the trough 2 wells 1 mm in diameter and equidistant from the central trough were also cut. A capillary tube was used to place the antigen in each of the wells, and the wells were filled to the brim. A small drop of Amido black was added to guage the completion of the run.

f) Setting Up the Run

Each slide was marked for the positive electrode. Three liters of buffer was placed in the immunoelectrophoresis chamber, and the slides were placed on rack in the chamber. Strips of filter paper, same width as the slide, were suspended in buffer and allowed to touch the edges of the slides on each slide. The power was set at 5 AMP/slide, and was run for 2½ hours. After the completion of the run, the trough was filled with mouse antiserum and incubated in a moist chamber at room temperature for 18-20 hours. The slides were then immersed in physiological saline for 24 hours, dried with filter paper strips, and immersed in Ponceau strain for 1 minute, and were immediately flushed with tapwater to remove excess stain. They were then dried and read.

5) Passive Cutaneous Anaphylaxis in Guinea Pigs

Guinea pigs of the Hartley strain weighing about 250-350 gm were used. The technique followed was that of Ormsbee et al. (1968) with slight modifications. 0.1 ml of mouse serum was injected intracutaneously into the shaven back of a guinea pig. At the end of approximately 24 hours 1 ml of the specific antigen in 0.5% Evans blue dye was injected intravenously into the saphenous vein. The reactions were then read after 30 minutes.

6) Passive Hemagglutination (PH)

Reagents

a) Diluent

Phosphate buffered saline (PBS) was prepared by adding 100 ml of saline to 23.9 ml of 0.15 M KH_2PO_4 and 76 ml of 0.15 M Na_2HPO_4, and the pH was adjusted to 7.2. In addition 100 ml of saline was added to 32.2 ml of 0.15 M Na_2HPO_4 and 67.7 ml of 0.15 M KH_2PO_4 and the pH was adjusted to 6.4. This diluent was prepared in 1 liter amounts, kept at 4°C, and was used throughout the experiment.

b) Tannic Acid

This solution was prepared by adding 5 mg of tannic acid powder to 50 ml of PBS, pH 7.2.

c) Red Blood Cells

Sheep red blood cells (rbc) collected in equal amounts of Alsevor's solution were obtained from National Biological Co. in Winnipeg, centrifuged at 1500 r.p.m. for 10 minutes, and the serum was discarded. The rbc were then washed three times with physiological saline, and a 6% solution of the rbc was prepared using PBS, pH 7.2.

Tanning the cells

Equal amounts of tannic acid solution and 6% solution of rbc were added together and mixed gently. The mixture

was then allowed to incubate at 37°C for 15 minutes. This was shaken occasionally and was then centrifuged at 1500 r.p.m. for 10 minutes, and the supernatant was removed and discarded. The cells were then washed with PBS of pH 6.4 once, and the original rbc concentration was made with the same PBS, pH 6.4.

d) Antigen

The antigen used was the same one that was employed in the Hemagglutination Inhibition experiment described previously. 1% of normal rabbit serum (NRS) was added to PBS, pH 6.4 to remove the Forssman antigen and the diluent was used to prepare the proper antigen dilution required. The proper antigen dilution (4 units/0.5 ml) was then added in equal amounts to the tanned cells, and the mixture was then mixed gently and incubated at 37°C for 30 minutes. The mixture was shaken once or twice during incubation, centrifuged at 1500 r.p.m. for 10 minutes and the supernatant was discarded. The mixture was washed three times with PBS, pH 6.4 with 1% NRS.

e) Serum

The serum used was that obtained from mice.

The Passive Hemagglutination Test

The test was performed according to the procedure

described by Stavitsky (1954) with some modifications.

One-fifth of a ml of PBS, pH 6.4 with 1% NRS was placed in each well, then 0.2 ml of the mouse serum was added to the first well, mixed, and half the volume was transferred to the second well; a two-fold dilution of the serum was made up to the dilution required. 0.2 ml of the sensitized tanned rbc was added to each well, and the mixture was allowed to stand for 30 minutes at room temperature and the plastic plate was placed at 4°C for a further 30 minutes.

Controls

Two controls were made:

1) two wells each contained 0.2 ml of sensitized tanned rbc and 0.2 ml of PBS, pH 6.4 with 1% NRS, but no serum

2) two wells contained 0.2 ml tanned but non-sensitized rbc, and 0.2 ml of the mouse serum.

7) Infectivity Neutralization Test

Agent

A DD strain of psittacosis propagated in eggs, and suspended in 50% skim milk, obtained from Dr. J.F. Winn, Beckman Laboratories, Chamblee, Georgia. The agent was estimated to contain an ELD_{50} of 5×10^6/ml.

Animals:

CF_1 strains of mice were used throughout the study; a preliminary experiment was performed to determine the dose to be used in mice to produce lesions with various degrees of mononuclear cellular infiltration.

For preliminary experiment four mice were anesthesized with ether, and were infected intratracheally through the oral route with half a ml syringes and 23 guage needles that were covered with plastic tubing to prevent any injury to the buccal mucosa. Three mice received 0.05 ml of 5×10^6 ELD_{50}/ml, and one mouse received 0.1 ml of the same dose. Each mouse was placed in a separate cage, and was checked daily for any deaths. After four days, the mice were sacrificed with ether, and each mouse was dissected in the neck region to expose the trachea. Two to three ml of Zenkers-formalin fixative solution (95% Zenkers solution and 5% of formalin solution) after the technique of Gogolak (1953) were injected into the lungs through the trachea using a 5 ml syringe and a 20 guage needle. The inflated fixed lungs were dissected out and placed in the same fixative solution for three hours. The lungs were washed for one hour with tap water and were placed in 70% alcohol solution and left overnight. The lungs were then placed in 95% alcohol (2 changes of half hour each); in 100% alcohol (4 changes of half hour each), into Toluene (2 changes of half hour each); in wax (2 changes of one hour each). The lungs were then embedded in wax and sectioned.

All staining and microscopic examinations of slides for histopathological findings and for inclusion bodies were performed at The Children's Hospital in Winnipeg through the help of Dr. H.L. O'Brien.

A three point scoring system was devised such that lung sections showing no lesions would be given a negative score, while those lungs which show force of lesions and cellular infiltration would be given a positive score. An intermediate score would be given to those sections showing only quite moderate infiltration of cells compared to those of the positive.

Staining The Lung Sections

1) The Harris Hematoxylin and Eosin Routine Stain

This was performed according to the method of Luna (1968).

Reagents

1) Acid alcohol: 1 litre of 70% alcohol with 10 ml of concentrated hydrochloric acid.
2) Ammonia water: 1 litre of tap water with 2-3 ml of 28% ammonium hydroxide.
3) Saturated lithium carbonate: 1 gm of lithium carbonate was added to 100 ml of distilled water and allowed to dissolve.

Staining procedure

Mercuric chloride crystals were removed from the sections and then were cleaned with sodium thiosulphate. Sections were placed in Harris' hematoxylin for 15 minutes, and then rinsed with water. The sections were differentiated by dipping them in the acid alcohol 3-10 times, and were checked to see the presence of distinct nuclei against colourless or light background, then they were washed in tap water briefly, and were dipped into ammonium water or lithium carbonate water until the sections were bright blue. The sections were washed in running tap water for 10-20 minutes, and were stained with eosin for 15 seconds to 2 minutes. The sections were dehydrated in 95% absolute alcohol until excess eosin was removed, and were placed in absolute alcohol, (two changes of 3 minutes each). The sections were placed in xylene (2 changes of 2 minutes each), and were mounted in Permount.

2) Wolbach's Giemsa Method

This was performed according to the method of Luna (1968). This stain was employed to reveal the presence of Inclusion bodies in the lung sections.

Reagents

1) Giemsa solution: this was prepared by the addition of 1 gm of Giemsa powder to 66.0 ml of glycerin and was placed in 60°C oven for 2 hours and

66.0 ml of methyl alcohol were added to the mixture. The working solution of this stock was made by the addition of 1.25 ml of the stock solution to 1.5 ml of methyl alcohol and 50.0 ml of distilled water.

2) Rosin alcohol solution: 10 gm of white Rosin was added to 100 ml of 100% alcohol. The working solution of this stock was made by the addition of 5.0 ml of the stock solution to 40.0 ml of 95% alcohol.

Staining procedure

The mercuric chloride crystals were removed from the sections and these were cleared with sodium thiosulphate. The sections were washed in running water for 15 minutes and were rinsed in distilled water; they were left in Giemsa solution for overnight. The next morning the sections were differentiated in Rosin alcohol solutions until sections assumed a purplish pink colour; were dehydrated in absolute alcohol and cleared in xylene (2 changes of each). The sections were then mounted with Permount.

The Infectivity Neutralization Test Proper

Sera

Mice sera obtained from groups A, B, (injected) and group C (infected) at different intervals of time were

sterilized using a millipore filter. 0.05 ml of the serum was added to 0.05 ml of 5×10^6 ELD_{50} of the agent in a plastic sterilized disposable CF plate. The mixture was incubated for 1 hour at 36°C and an individual mouse was used for each separate mixture. Each mouse was then placed into a separate cage, and left for 4 days. The mice were sacrificed at 4 days after inoculation and their lungs were fixed and stained in the same manner as described above. The degree of neutralization found was read according to the reduction of size and number of lesions produced by the agent in the lung tissue.

RESULTS

1) Complement Fixation (CF) Test

In group A mice a CF titer of 1:8 was observed on the third and sixth day (Fig. 1). The titer then rose and reached a peak of 1:32 on the twelfth day and remained at this level up to the eighteenth day. The CF titer th n dropped in a consistent manner but slowly until on the fifty-eighth day it became less than 1:4. Serum collected on the forty-third day showed anti-complementary activity and no CF titer could be determined.

In group B mice which received twice as much dose of the antigen as group A mice, the serum showed a titer of less than 1:4 at 3 days (Fig. 2) but rose consistently reaching the level of 1:32 at the twelfth day, and remained at that level until the twenty-first day. On the twenty-first day, mice in this group received a booster of the same antigen which they received initially. A titer of 1:64 was recorded on both of the twenty-fourth and thirty-third days. On the thirty-eighth, forty-third and forty-eighth day a titer of 1:32 was observed. On subsequent days a consistent decline was seen in the titer until it reached a level of 1:8 on the fifty-eighth day.

In group C mice which received infectious agent showed a titer of less than 1:2 at two days (Fig. 3) but it rose

consistently reaching a peak of 1:16 on the thirteenth day. The same titer was recorded on the fifteenth day. Subsequent reading on the twenty-second day showed a titer of 1:12.

2) Hemagglutination Inhibition (HI) Test

In group B mice the titer rose to 1:20 on the nineteenth day (Fig. 4) but fell to 1:10 on the twenty-second day. The titer then rose to 1:320 on the thirty-third day and remained at this level for approximately one week, then the titer dropped consistently to less than 1:10 on the forty-third day, and no titer was observed on the fifty-third and fifty-eighth day.

In group C mice which was infected orally, the HI titer rose to 1:10 on the fourth day after infection (Fig. 5) and rose steadily to 1:80 on the sixth day and reached its peak of 1:320 on the eighth day. The titer dropped consistently to 1:80 on the thirteenth day and its lowest value of 1:20 on the fifteenth day.

3) Macrophage Spreading Inhibition (MSI) Test

In group C mice an average MSI index of 96.40 was recorded on the ninth day. It dropped to 63.75 on the fifteenth day and rose again to 93.80 on the twenty-first day (Fig.6).

In group M which received non-infectious agent showed an MSI index of 64.50 on the tenth day, which dropped to 60.75 on the seventeenth day and rose to 78.00 on the twenty-fourth day (Fig. 7).

4) Immunoelectrophoresis

Serum from group A mice was fractionated by sucrose density gradient and immunoelectrophoresis was carried out on fractions collected on the sixth, eighteenth and thirty-third days. Two peaks of CF activity were observed between eluates 1.5 and 2.5, and between 3 and 4.5 in each of the fractions collected on the sixth, eighteenth and thirty-third days. The two peaks corresponded with 7S and 19S immunoglobulins.

On the sixth day the first peak showed a CF titer of 1:256, while the second peak showed a CF titer of 1:512 (Fig. 9). On the eighteenth day both the peaks showed similar CF reactivity with a titer of 1:128, (Fig. 10) while on the thirty-third day the first peak showed a CF titer of 1:32 and the second peak showed a CF titer of 1:16 (Fig. 11).

5) Passive Cutaneous Anaphylaxis (PCA) Test

Group A mice showed no PCA reactivity (Table 9). Group B showed a positive PCA reactivity on the 29th day,

but no reactivity on subsequent tests. Group C showed no reactivity on the second, fourth, sixth and thirteenth days, but gave a positive reaction on the eighth and fifteenth day. The controls (group Z) were negative on both the thirty-eighth and fifty-eighth day.

6) Passive Hemagglutination (PHA) Test

The passive hemagglutination titers on sera from group B mice showed a titer of 1:32 on the third day (Fig. 12). This rose to 1:64 by the sixth day, but declined to 1:16 on the ninth day, with a subsequent rise to 1:32 on the twelfth day. On the fifteenth day the titer dropped to 1:4.

7) Infectivity Neutralization Test

Results of infectivity neutralization tests carried out on sera from mice of group A, C and Z are given in Table 11. Sera from group A mice showed protective activity on the thirty-eighth, forty-third and fifty-fourth and fifty-ninth day, since no inflammatory reaction, nor any mononuclear cellular migration could be observed in the lung sections. Sera from the thirty-third, and forty-eighth days showed no protective activity. In mice receiving thirty-three day sera numerous and diffuse coalescent inflammatory foci were observed involving the entire lobes of lung tissue. Consolidation, and presence of intraalveolar

edema in the adjacent section, with fibrin deposition and haemorrhage was also noted. In mice receiving the forty-three day serum large foci of inflammation encompassing nearly the entire lung sections were seen (Fig. 13 and 14). Similar reactions were observed in the sections of the lungs of mice receiving forty-eighth day serum.

The sera from group C showed positive activity on the sixth day, while the rest gave no protection.

TABLE 4

Results of Complement Fixation tests on pooled sera from mice of groups A and B injected subcutaneously with 0.5 ml containing 0.5 mg and 1 mg of N_2/ml respectively, and group Z injected with normal allantoic fluid.

Days after Injection	TITER*		
	Group A	Group B	Group Z
3	8	<4	
6	8	8	
9	16	16	
12	32	32	
15	32	32	
18	32	32	
21	16	32	
24	16	64	
33	16	64	
38	8	32	0
43	AC(+)	32	
48	4	32	
53	<4	16	
58	<4	8	0

* expressed as reciprocal of serum dilution

(+) Anticomplementary

TABLE 5

Results of Complement Fixation on pooled sera from group C mice infected orally with 0.05 ml of 10^{-3} MLD_{50}.

Days after Infection	Titer*
2	4
4	4
6	4
8	8
13	16
15	16
22	12

* expressed as reciprocal of serum dilution

TABLE 6

Results of Hemagglutination Inhibition test on sera from group B mice injected intraperitoneally with 0.5 ml containing 1 mg/ml of N_2, and group Z mice injected with normal allantoic fluid.

Days after Injection	Titer*	
	Group B	Group Z
19	20	
22	10	
29	320	
33	320	
38	80	negative
43	80	
48	<10	
53	negative	
58	negative	negative

* Titers expressed as reciprocal of dilution.

TABLE 7

Results of Hemagglutination Inhibition test on sera from group C mice infected orally with 0.05 ml of agent containing 10^{-3} MLD_{50}/ml.

Days after Infection	Titer*
4	10
6	80
8	320
13	80
15	20

* Titers expressed as reciprocal of dilution.

TABLE 8

MSI index on mice from group C infected orally with 0.05 ml of 10^{-3} MLD_{50} and mice from group M injected in the foot pad with 2 mg of N_2/ml.

	Days after Inoculation	No. of Mice	Average of MSI	Range
Infected	9	5	96.40	82 - 100
	15	4	63.75	44 - 82
	21	5	93.80	78 - 100
Injected	10	4	64.50	57 - 75
	17	4	60.75	50 - 80
	24	4	78.00	61 - 100

TABLE 9

Results of Passive Cutaneous Anaphylaxis reactivity in sera from mice of Groups A, B, C and Z.

Group	Days after Injection or Infection	PCA Reactivity
A	22	-
	38	-
	43	-
	48	-
	53	-
	58	-
B	29	+
	33	-
	43	-
	48	-
	53	-
	58	-
C	2	-
	4	-
	6	-
	8	+
	13	-
	15	+
Z	38	-
	58	-

TABLE 10

Results of Complement Fixation and Passive Hemagglutination tests on pooled sera from group B mice injected subcutaneously with 0.5 ml containing 1 mg of N_2/ml.

Days after Injection	CF titer*	PH titer
3	4	32
6	8	64
9	16	16
12	32	32
15	32	4

* expressed as reciprocal of serum dilution

TABLE 11

Results of Infectivity Neutralization tests on sera from mice of groups A, C and Z.

Group	Days after Injection or Infection	Reactivity of Serum*
A	22	Dead
	29	Dead
	33	-
	38	+
	43	+
	48	-
	54	+
	59	+
C	2	Dead
	4	-
	6	+
	8	Dead
	13	Dead
	15	-
Z	38	Dead
	59	Dead

* Dead refers to the fact that the serum had no neutralizing activity and the animals died of infection; - refers to inability of serum to neutralize the agent but animals survived; + refers to ability of serum to neutralize the agent.

Figure 1

Complement Fixation titers of sera from mice of group A injected subcutaneously with 0.5 ml containing 0.5 mg/ml of N_2.

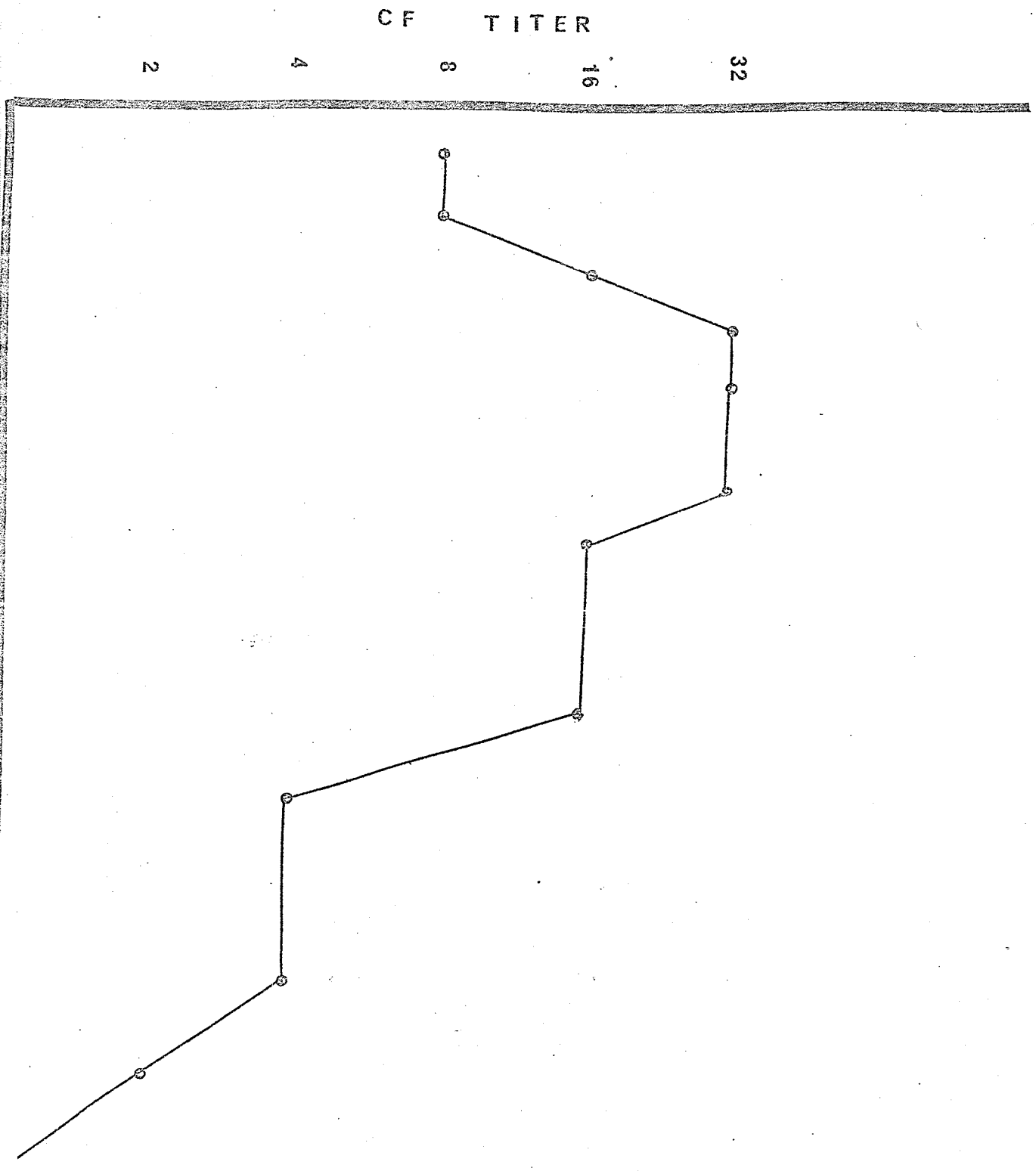

CF TITER
2
4
8
16
32

Figure 2

Complement Fixation titers of sera from mice of group B injected subcutaneously with 0.5 ml containing 1 mg/ml of N_2.

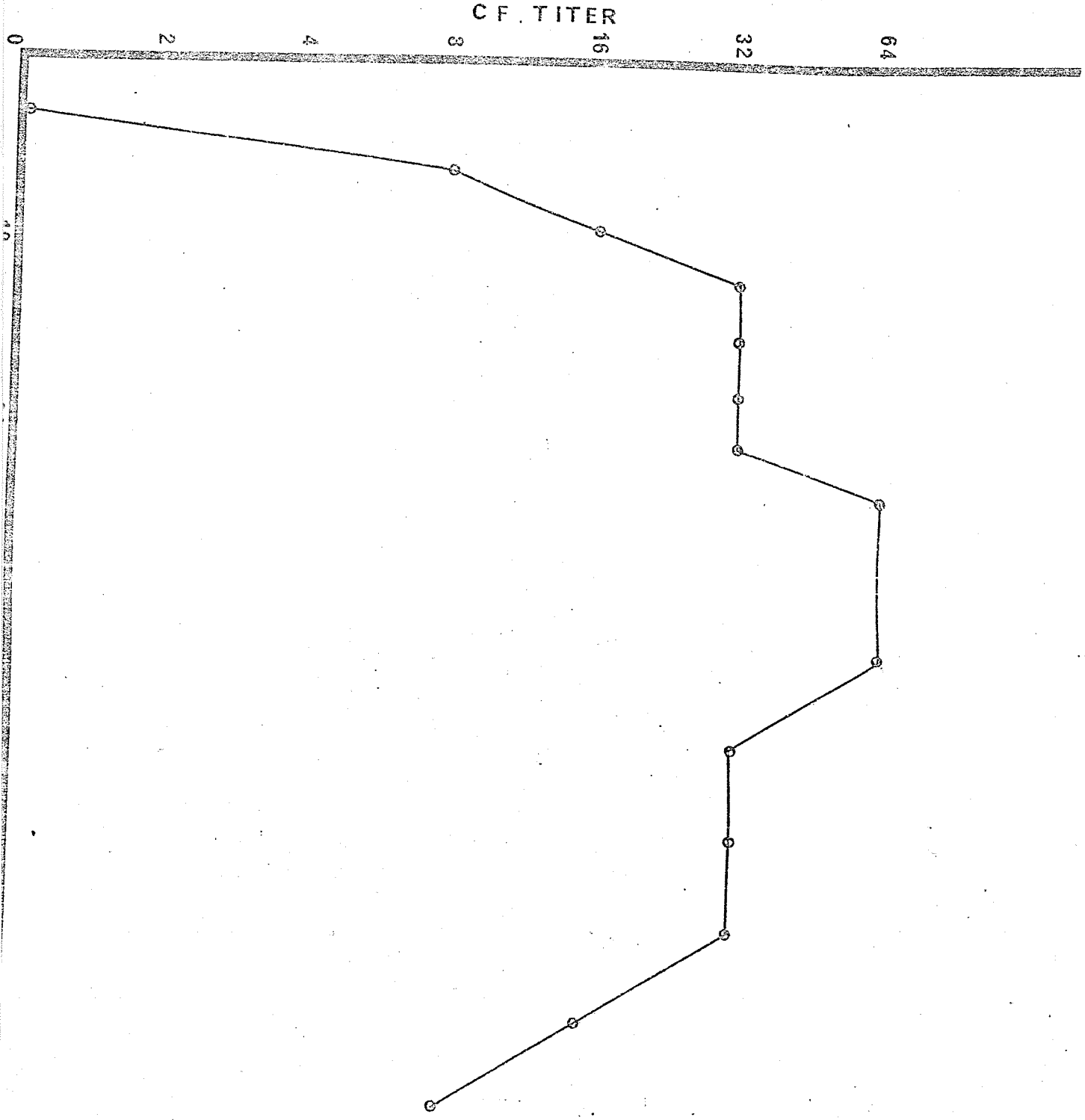

C F . TITER
0
2
4
8
16
32
64

Figure 3

Complement Fixation titers of sera from mice group C infected orally with 0.05 ml of 10^{-3} MLD_{50}.

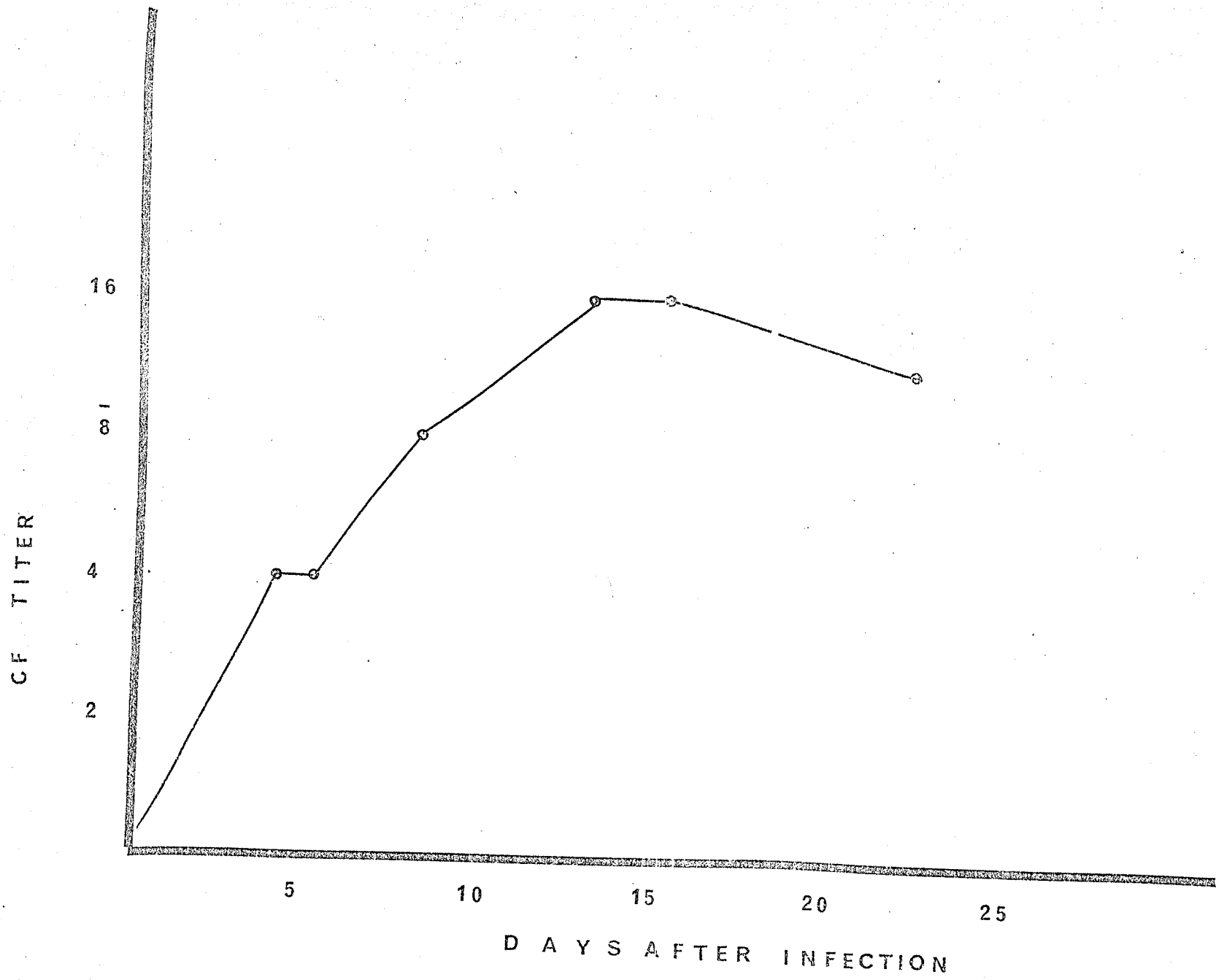

16
8
4
2
CF TITER
5
10
15
20
25
DAYS AFTER INFECTION

Figure 4

Results of Hemagglutination Inhibition test on sera from group B mice injected intraperitoneally with 0.5 ml containing 1mg/ml of N_2.

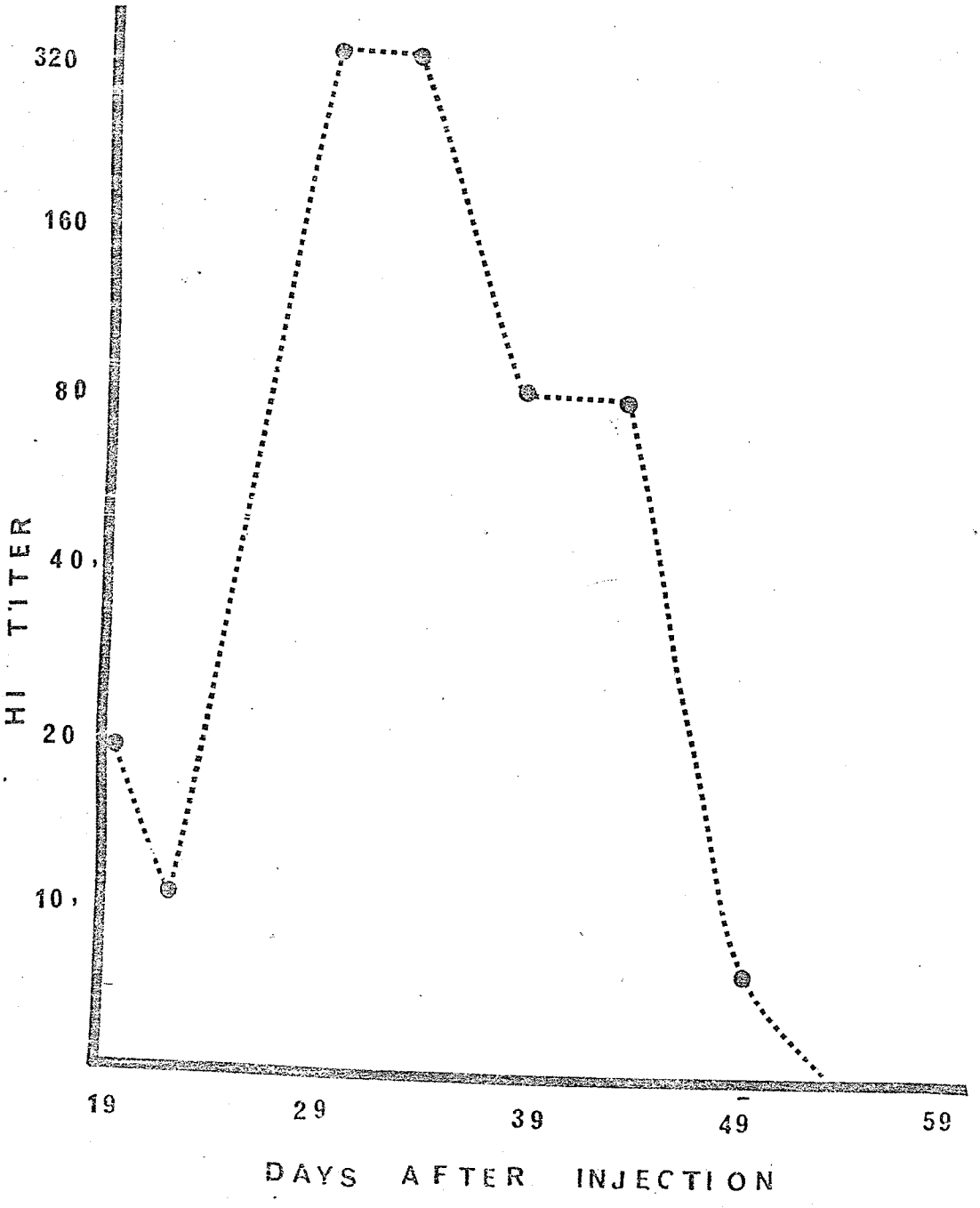
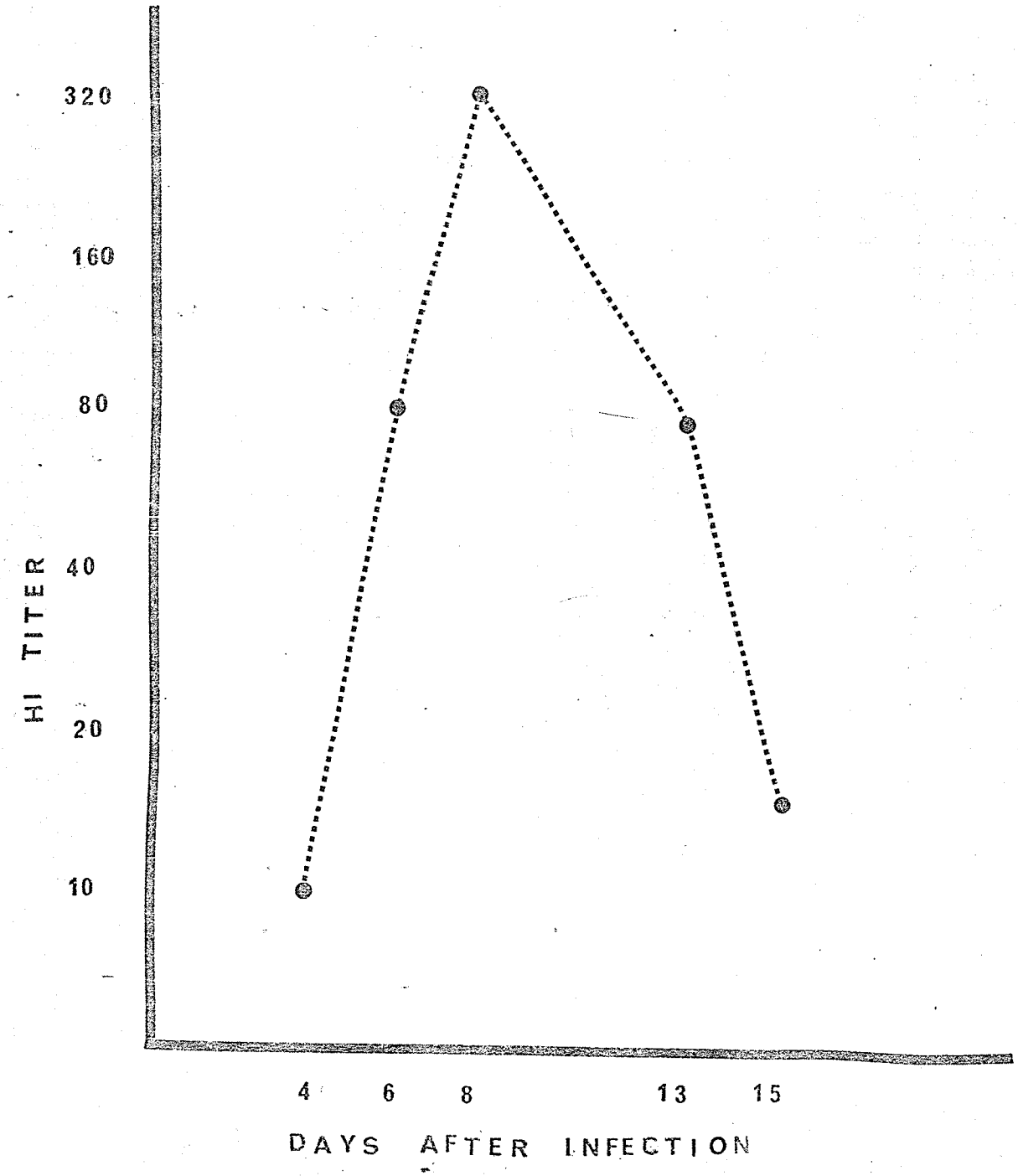

320
160
80
40
20
10
HI TITER
19
29
39
49
59
DAYS AFTER INJECTION

Figure 5

Results of Hemagglutination Inhibition test on sera from group C mice infected orally with 0.05 ml of agent containing 10^{-3} MLD_{50}/ml.


320
160
80
40
20
10
HI TITER
4
6
8
13
15
DAYS AFTER INFECTION

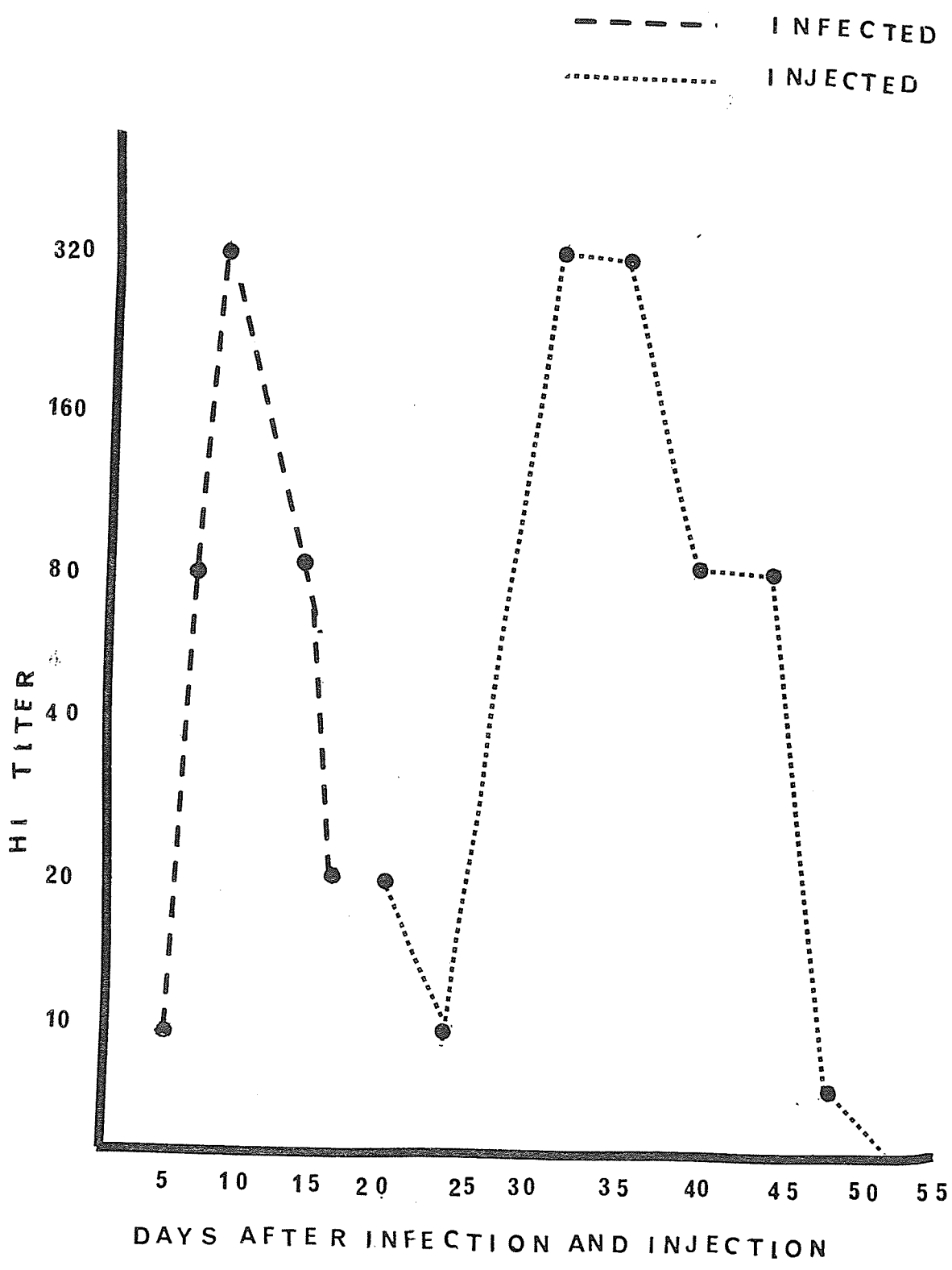

INFECTED
INJECTED
320
160
80
40
20
10
HI TITER
5
10
15
20
25
30
35
40
45
50
55
DAYS AFTER INFECTION AND INJECTION

Figure 6

MSI Index from group C mice infected orally with 0.05 ml of 10^{-3} MLD_{50} at 9, 15, and 21 days after infection.

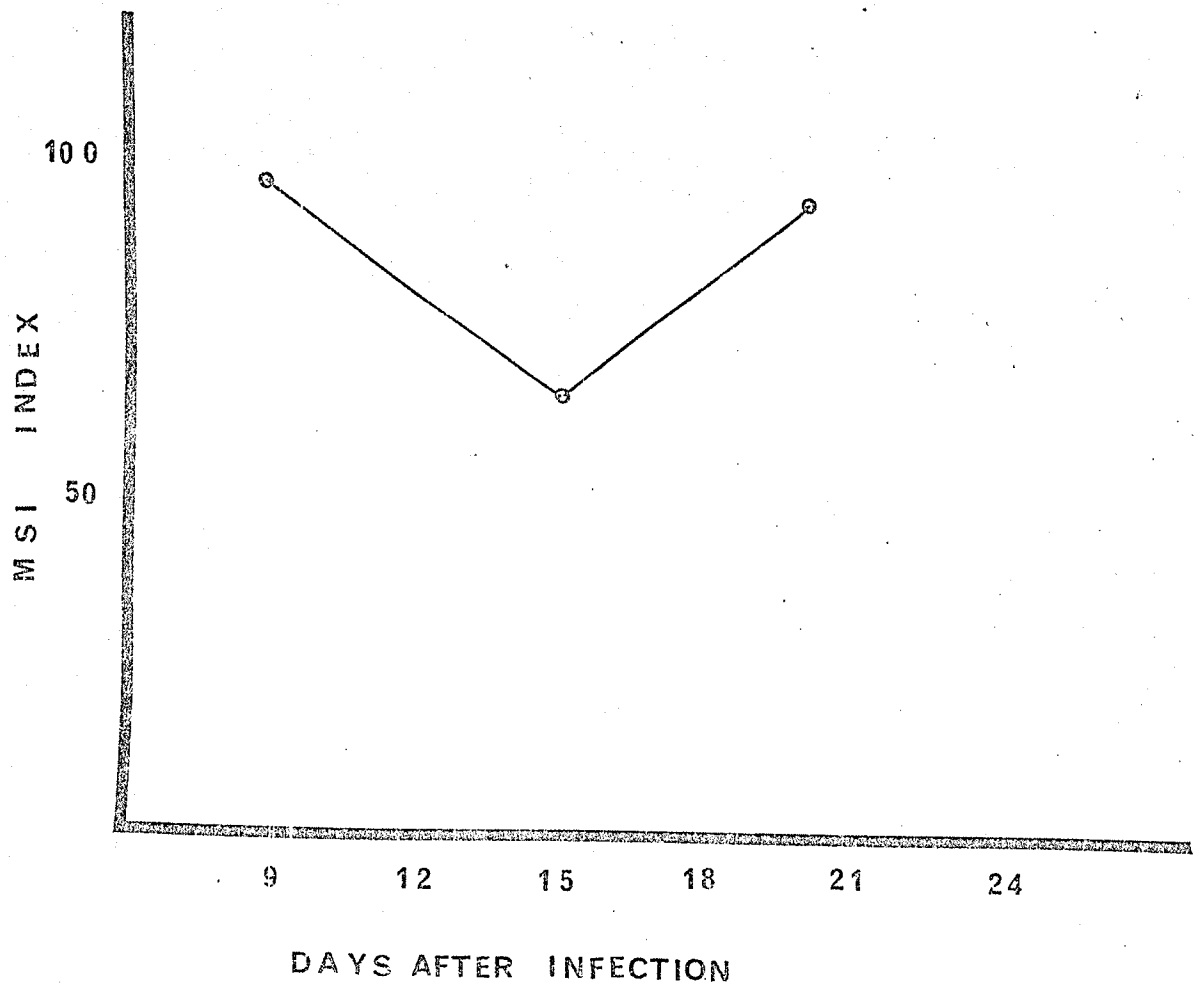

100
50
MSI INDEX
9
12
15
18
21
24
DAYS AFTER INFECTION

Figure 7

MSI Index from group M mice injected in the foot pad with 0.05 ml containing 2 mg/ml of N_2 at 10, 17 and 24 days.

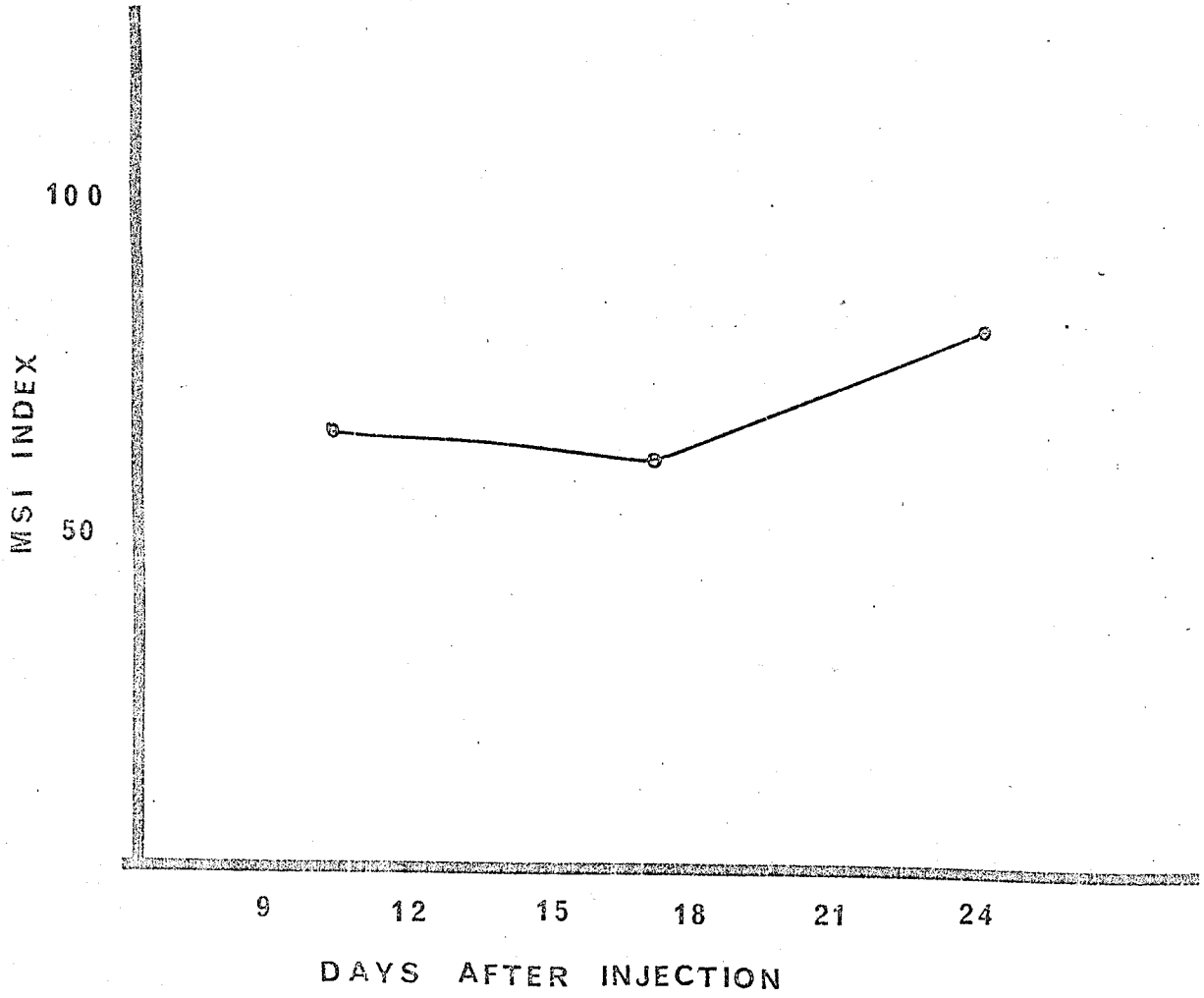

100
50
MSI INDEX
9
12
15
18
21
24
DAYS AFTER INJECTION

Figure 8

Comparison of MSI Indices from group C mice infected orally and mice from group M injected in the foot pad.

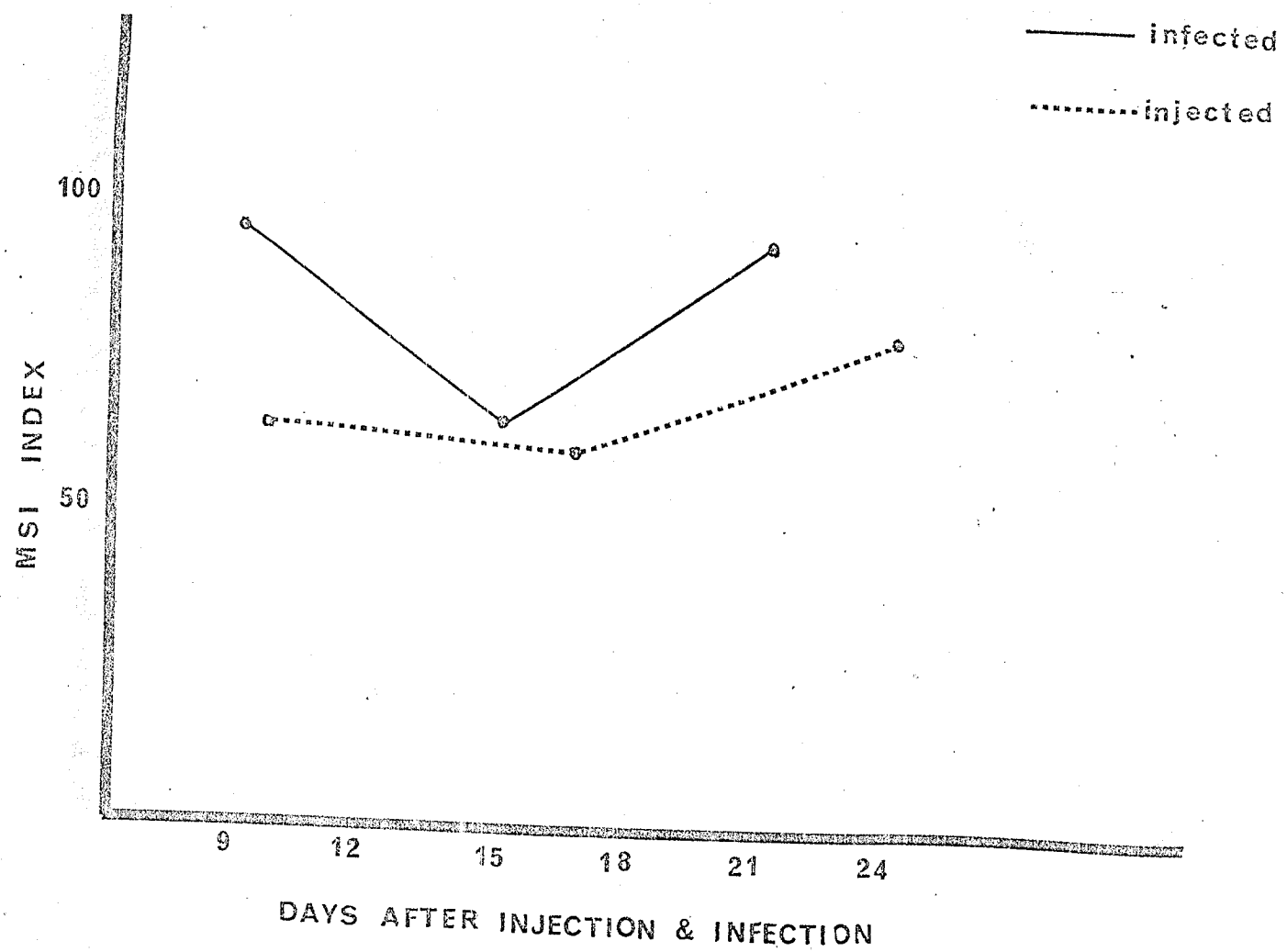

infected
injected
100
50
MSI INDEX
9
12
15
18
21
24
DAYS AFTER INJECTION & INFECTION

Figure 9

Complement Fixation and Immunoelectrophoresis of sucrose density gradient fractions of pooled mice serum at 6 days from group A injected subcutaneously with 0.5 ml containing 0.5 mg/ml of N_2.

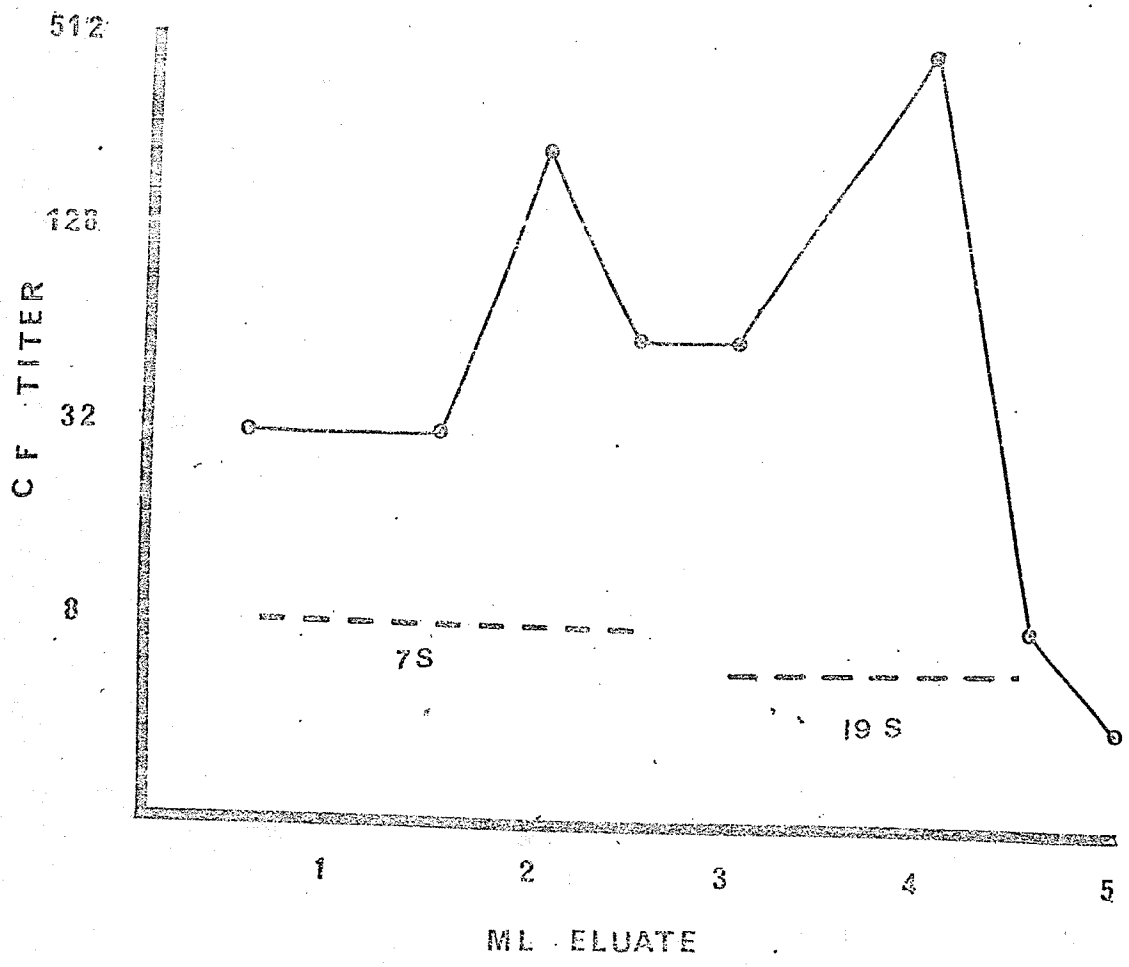

512
128
32
8
CF TITER
7S
19 S
1
2
3
4
5
ML ELUATE

Figure 10

Complement Fixation titer and Immunoelectrophoresis of sucrose density gradient fractions of pooled mice serum at 18 days from group A injected subcutaneously with 0.5 ml containing 0.5 mg/ml of N_2.

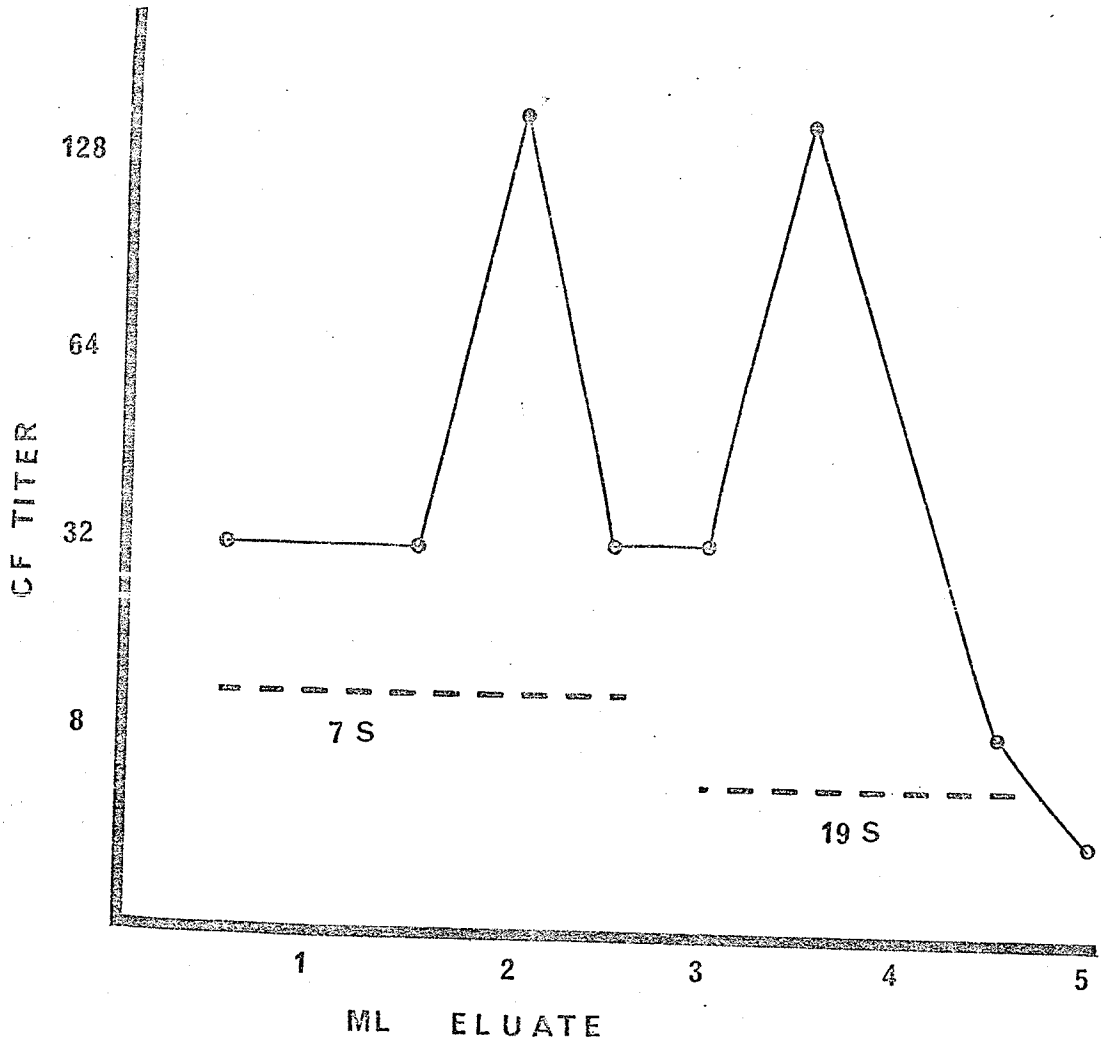

128
64
32
8
CF TITER
7 S
19 S
1
2
3
4
5
ML ELUATE

Figure 11

Complement Fixation and Immunoelectrophoresis of sucrose density gradient fractions of pooled mice serum at 33 days from group A injected subcutaneously with 0.5 ml containing 0.5 mg/ml of N_2.

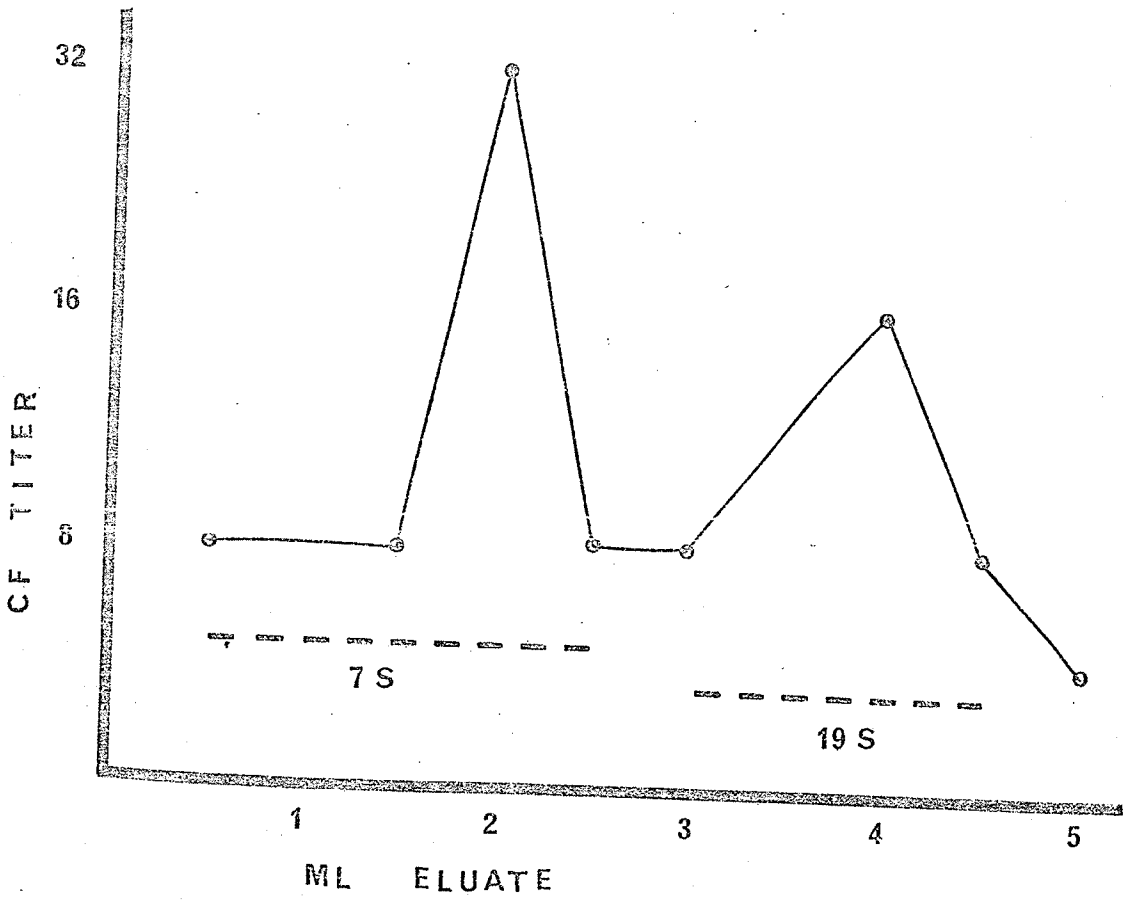

32
16
8
CF TITER
7 S
19 S
1
2
3
4
5
ML ELUATE

Figure 11(a)

Comparison of Results of Complement Fixation and Immunoelectrophoresis of sucrose density gradient fractions of group A pooled mice sera at 6, 18, and 33 days.

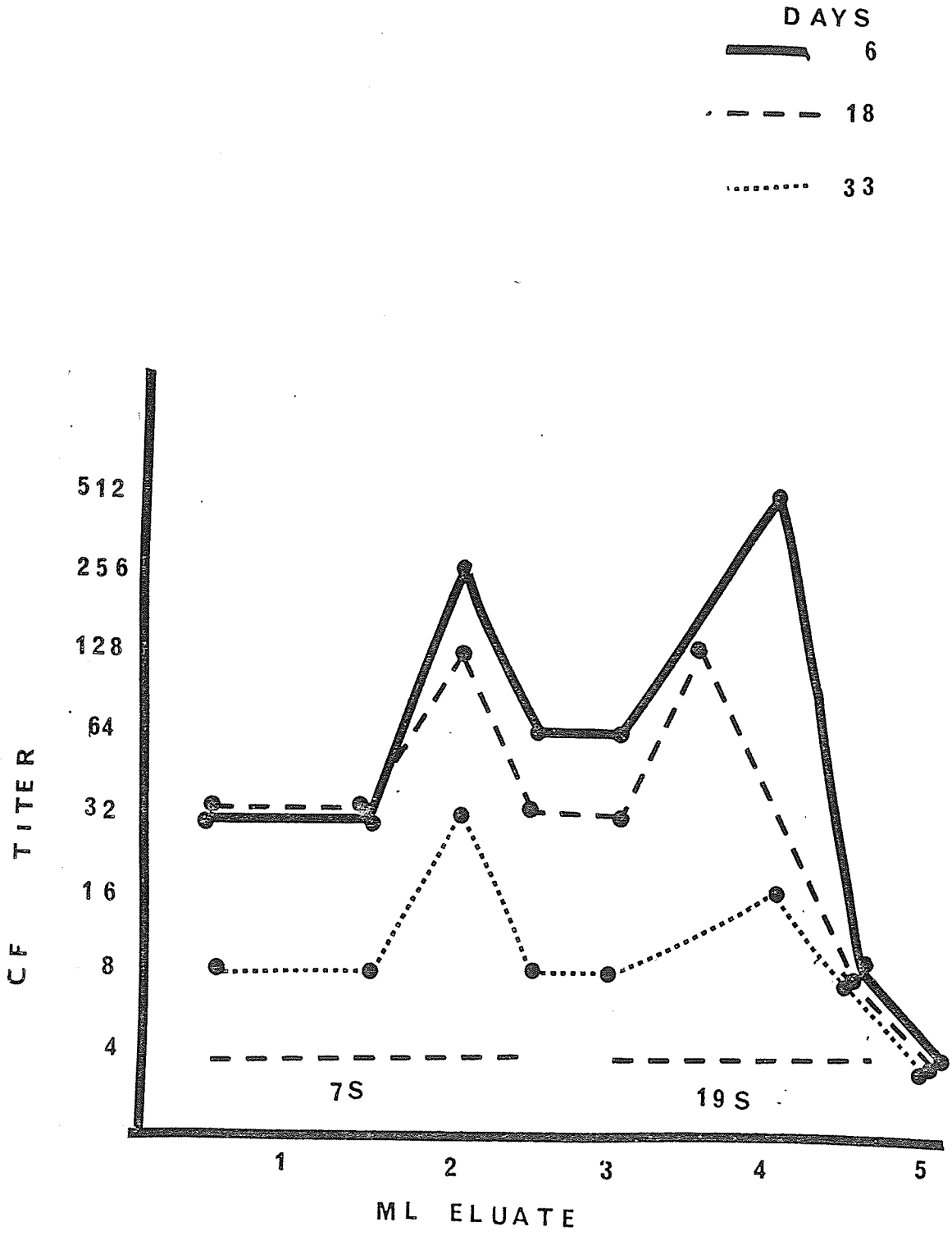

DAYS
6
18
33
512
256
128
64
32
16
8
4
CF TITER
7S
19S
1
2
3
4
5
ML ELUATE

Figure 12

Comparison of the Passive Hemagglutination and Complement Fixation titers in group B mice injected with 0.5 ml containing 1 mg/ml of N_2.

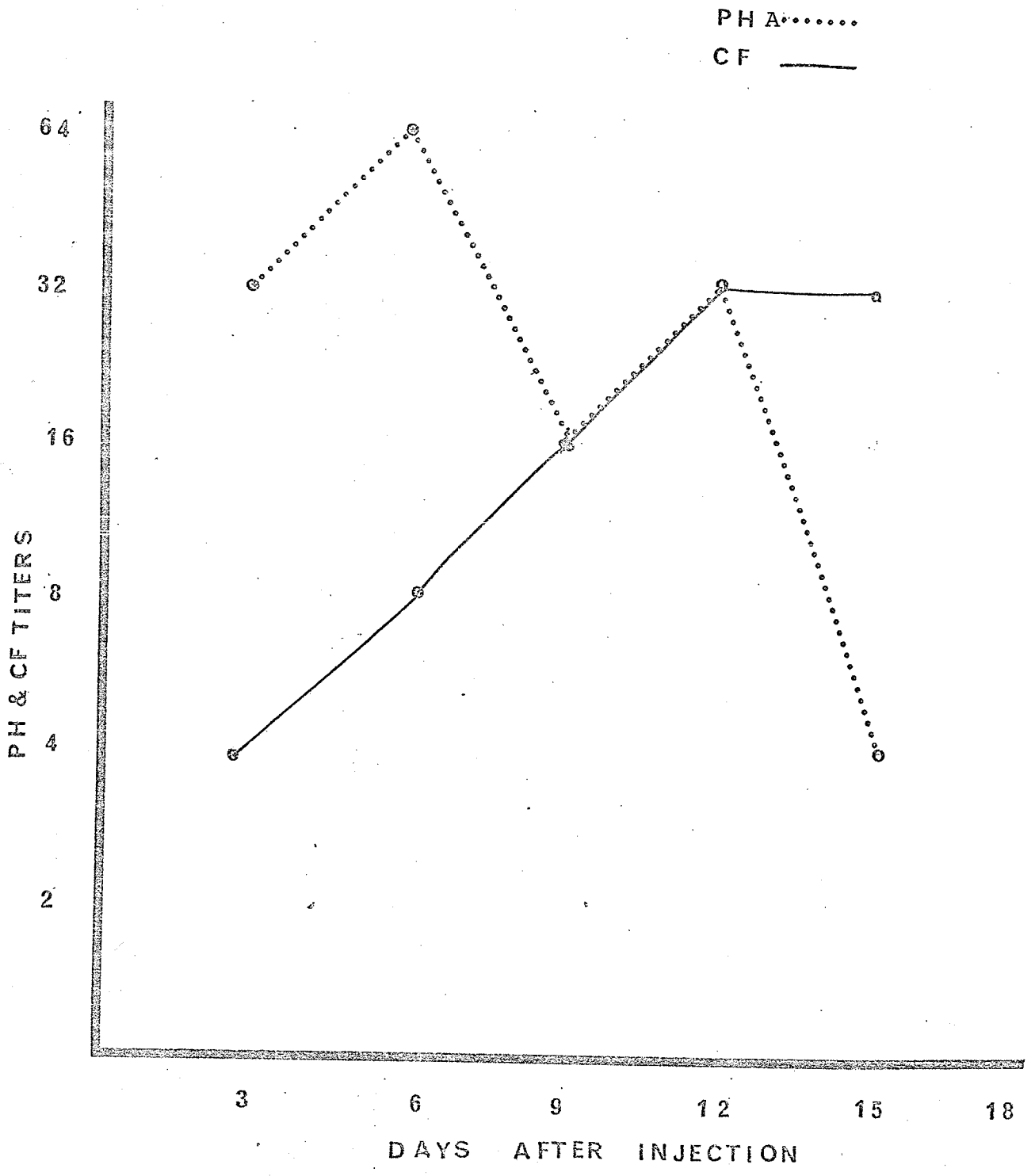

PH A.....
CF ——
64
32
16
8
4
2
PH & CF TITERS
3
6
9
12
15
18
DAYS AFTER INJECTION

Figure 13

A photomicrograph of a mouse lung section stained with Hematoxylin and Eosin with 12x magnification four days after infection showing extensive inflammatory consolidation involving portions of most lobes.

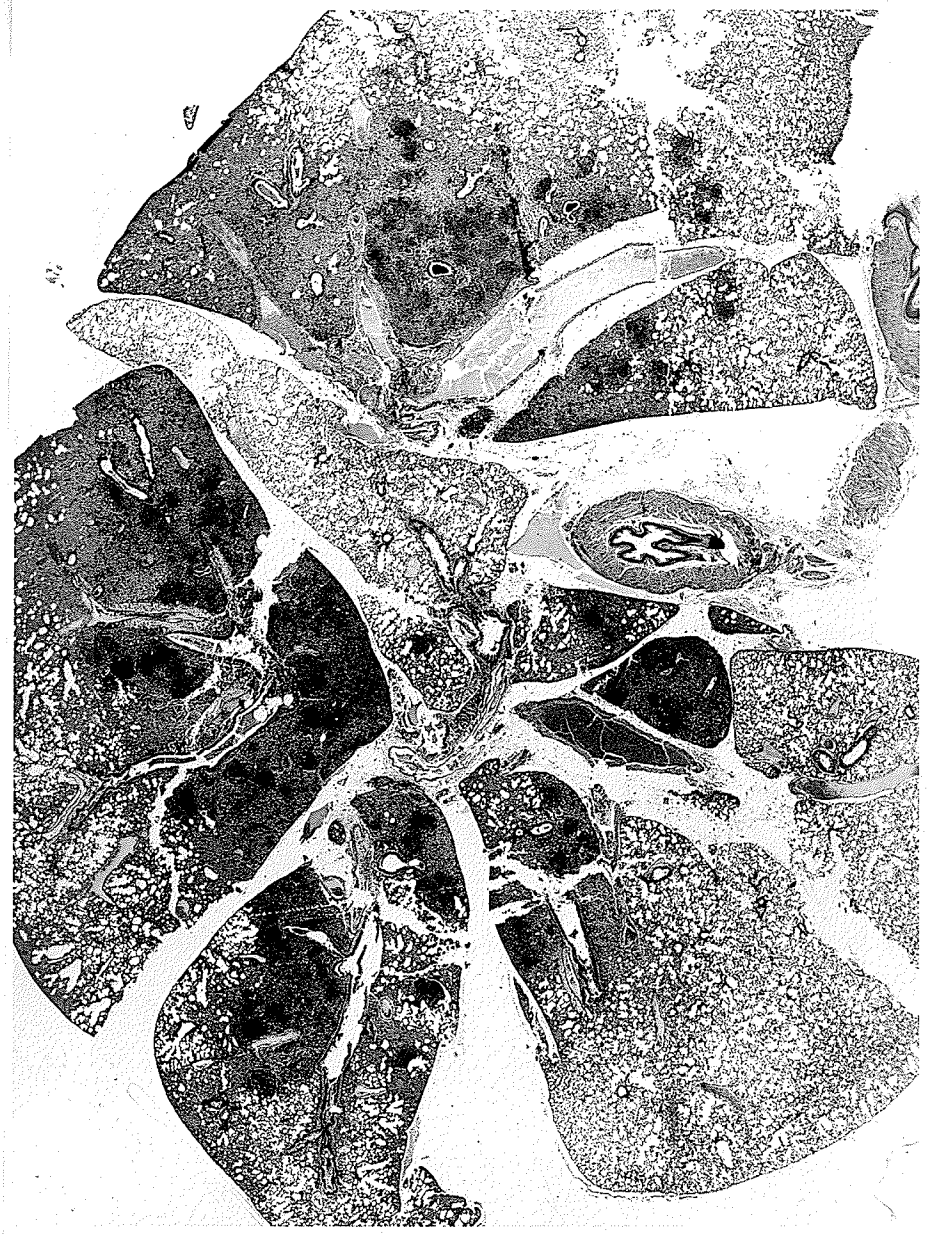
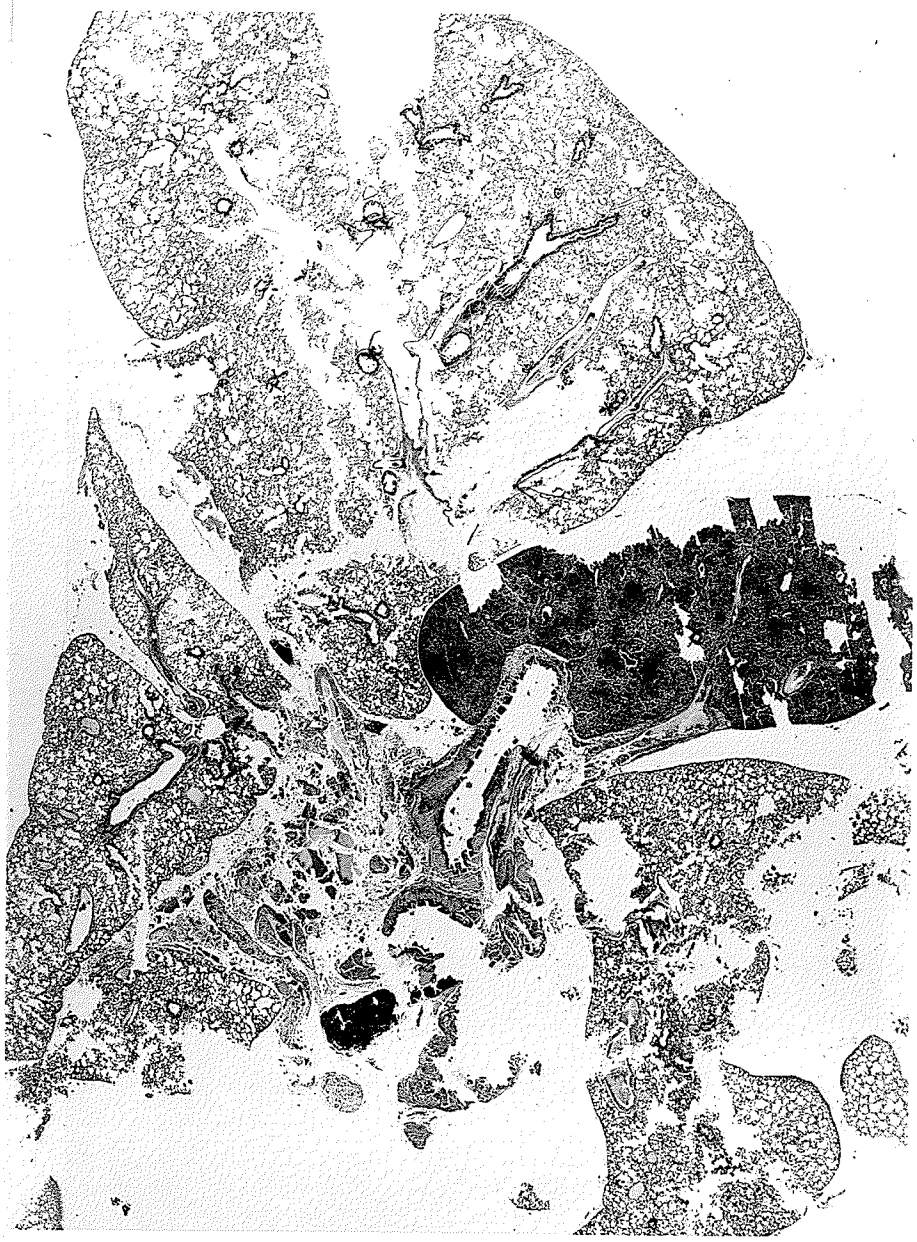


Figure 14

A photomicrograph of a mouse lung section stained with Haemotoxylin and Eosin with 12x magnification four days after infection showing inflammatory consolidation of only one lobe (the dark area). The light area shows normal lung tissue.



DISCUSSION

The results of CF experiment show that in group A mice the titer rose sharply from 1:8 at the ninth day to the highest titer of 1:32 on the twelfth day. This titer was maintained for about one week after which it dropped slowly but in a consistent manner until the end of the two month period, where a titer of less than 1:4 was reached. Since the agent used for immunization was made non-infectious by repeated washings with phosphate buffer, and not by chemical methods, a residual infectivity of about one percent was present in the immunizing agent. This residual infectivity may have been responsible for repeated stimulation of the antibody producing cells, resulting in several periods of approximately one week duration during which the titer maintained its level. The CF titer remained at 1:16 from the twenty-fourth to the thirty-third day after immunization, then dropped to 1:4 on the thirty-eighth day and continued at this level up to the fourth-eighth day after immunization. The magnitude of the residual infectivity caused no rise in the titer which was maintained at the same level.

The results obtained from sera of group B mice, which received twice the dose given to group A revealed a similar sharp rise in the titer. A titer of 1:32 was attained at the twelfth day and was maintained for approximately one week. The observed rise in the titer to 1:64 was due to the

booster dose of the immunizing agent which was given to this group at 21 days after the initial injection. This high titer was maintained for approximately 12 days, then dropped to 1:32 on the 38th day and remained at that level until the 48th day. The titer then dropped consistently to 1:8 on the 58th day. It was also noted that the lowest titer obtained in group B was 1:8 by the end of the experimentation period while that in group A was less than 1:4. These observations clearly show that doubling the immunizing dose increases the titer level and that a booster dose results in a further increase.

The results obtained from sera of group C mice show a different pattern. The titer rose to 1:16 on the 13th day and was maintained at the same level for a short period of 2 days after which it dropped to the level of 1:8. Since group C mice was infected orally, several mice died because of this infection. The low titer of 1:16 attained may have been due to different factors inherent to the immune response of the host on the antigenic ability of the infectious agent.

These results are not fully in accord with those of other studies. Graber and Pomeroy (1958), reported that the CF titer in poultry workers infected with psittacosis persisted at a high level (1:8 to 1:128) for up to 32 months. Similarly Matthiesen and Volkert (1956) observed a high titer of 1:60 which persisted for 20 months. The results of this study, however, show a drop in the titer to less than 1:4 within two months from the time of immunization. The maintainance

of a moderately high titer for such long periods of time may have been due to repeated infections in the poultry workers. The host response (e.g., man vs. mice) to the agent may also be a significant factor resulting in this discrepancy.

When sera of group B mice was tested for the presence of specific antibodies to the hemagglutinating antigen, a titer of 1:20 was observed on the 19th day after immunization, and dropped to 1:10 on the 22nd day. This slight drop of titer could be due to the disappearance of the specific hemagglutinating antigen that triggers the specific HI sera. The booster dose given on the 21st day caused an instantaneous rise in the titer to a peak of 1:320 which persisted for less than a week, and then dropped consistently to zero titer around the 53rd day. The oral infection of group C mice caused their titer to rise to a peak of 1:320 by the end of the first week. This titer, however, dropped to a level of 1:20 by the end of the second week after infection. The persistance of this level could not be estimated due to the premature termination of this experiment. This pattern may be similar to the pattern of natural infection.

The highest MSI index was achieved in both the infected and injected group C and M at approximately 14 days after treatment. These results are in accord with those reported by Nicks and Sayed (1970). The lowest values for the MSI

index obtained around the second week for groups C and M were 63.75 and 60.75 respectively. The significance of these two values in arriving at a definite conclusion as to the presence of delayed hypersensitivity may require further investigations.

The results of the serologically active antibodies observed using the immunoelectrophoresis technique indicate that the CF reactivity tends to be concentrated in two peaks which are concentrated in the 19S and 7S fractions of immunoglobulins, this activity tends to drop with time, and there is a shift in this activity from the 19S protein to the 7S protein when results are compared between six days and thirty-three days after immunization.

The detection of a skin reaction in guinea pigs, using the PCA technique, with only one booster dose indicates the presence of allergic type antibody within the first month of injection. Ormsbee et al. (1968) inspite of repeated immunizations were not able to observe the PCA reactivity until the eightieth day of infection. This may be due to the use of the Rickettsial antigen rather than pisttacosis employed in this study. The detection of one skin reaction one week after infection of group C mice, and another on the fourteenth day, may be to the live agent which was able to induce the formation of immunoglobulins responsible for the allergic reactions. The component of the agent responsible

for the production of PCA antibodies might have been denatured in the non-infectious material which was used to immunize the non-infected groups A and B. It may be speculated that, since, in nature man acquires the infection through the respiratory tract, a similar response to that observed in group C may be expected.

Since the PHA technique measured higher titer than the CF during the first two weeks after immunization, it may be concluded that the PHA is the more sensitive technique in that period. The CF technique however is considered to be the more reliable technique due to its specificity to more serologically defined antigens, while the antigens used in the PHA tests could be derived from any part of the agent and are consequently different according to the method of their preparation.

The immune sera for the infectivity neutralization test were not collected until one month after immunization. This decision was reached based on the works of Hilleman (1945), Moulder (1958), and St. John and Gordon(1947). These workers reported that most of the protective and neutralizing antibody appear approximately one month after immunization. The results of the infectivity neutralization tests revealed continuous protection as indicated by tests performed on 38th, 43rd, 54th and 59th days. The inability of the serum tested on the 48th day to neutralize the agent could not be explained. It should be noted however, that the protection

was in all other cases complete and no inflammatory lesions or consolidation in the lung sections were detected. In contrast prior to the 38th day, when no neutralization could be detected, the inflammatory response produced encompassed as much as 70% of the total lung sections.

The ability of the sera obtained from the infected group on the sixth day after infection to neutralize the agent could not be explained on the basis of this study. However, the presence of neutralizing antibodies in natural infections of humans for longer periods of time merits further investigation.

The results obtained in the injected groups indicate that both the complement fixing and hemagglutination inhibiting antibodies showed their highest peak at approximately 30 days after immunization. The complement fixation titer however reached the peak of only 1:64, while the hemagglutination inhibition titer reached the peak of 1:320.

The drop in the hemagglutination inhibiting antibodies was faster than that of the complement fixing antibodies. At sixty days after immunization, no hemagglutination inhibiting antibody titer was detected while the level of complement fixing antibodies was still as high as 1:8. The degree of persistance of this titer beyond the 60th day after immunization may not be assessed.

In contrast, peak titers reached in the sera obtained from the infected group was as early as seven days after

infection. The level of the hemagglutination inhibition antibodies (1:320) was similarly higher than that of the CF (1:16). The drop in both titers was comparable, both maintaining a level of approximately 1:10 by the end of fifteen days. The degree of the persistence of the titers beyond this time could not be investigated due to severe infections, and a high incidence in the mortality of animals.

In conclusion the results of these investigations indicate that both the complement fixation and hemagglutination inhibition technique can be adequately utilized for diagnosis in humans. High titer levels revealed in human sera by the two techniques combined may give an indication of an early infection since the time of infection is not usually known. Either of the two techniques is adequate within the first 30 days of infection, but the complement fixation technique seems more reliable up to at least sixty days. Other investigators, Graber and Pomeroy (1958) have demonstrated the adequacy of the complement fixation technique for up to 32 months. However it should be considered that the immune response to chlamydial agents might be different in different host species.

The cellular immunity revealed by the macrophage spreading inhibition technique in both the infected and injected mice was similar. This suggests the use of a skin test for psittacosis disease, as a supportive evidence for the presence of an infection to the chlamydial agent, in a manner similar to the Mantoux survey for a previous

exposure to the Mycobacterium tuberculosis or related agents.

The observed results on the serological reactivity of immunoglobulins tested by immunoelectrophoresis pointed out that the specific complement fixing antibodies against chlamydial agent reside in the 7S and 19S proteins. The concentration of the complement fixing antibodies after six days in the 19S protein and then the presence of a higher concentration of complement fixing antibodies in the 7S protein after thirty days seem to agree with data found by Sayed and Wilt (1971) who found no complement fixing activities in the supernates of fractions which contained IgM and IgG antibodies when these fractions were absorbed by the psittacosis agent.

The allergic antibodies were shown to be present on the 29th day after immunization of the injected group (group B) as well as one and two weeks after the infection in group C. The extent to which such antibodies react in favour or against the host and their contribution to the severity of the histopathological lesions in human infections merit further investigations.

The results of infectivity neutralization tests on the sera from immunized mice indicated the presence of protective antibodies thirty days after immunization. This protection persisted for at least thirty days. Further persistance of such protection was not assessed.

The degree of protection which such antibodies confer

to humans may differ from that in mice. Although it was in mice adequate, the antibodies produced in humans may contribute to the chronicity and latency of the disease. Mice infected by Yanamura and Meyer (1942) passed through latent infections and became persistent carriers for up to 425 days. As observed by Meyer and Eddie (1962) humans may be carriers of the disease without showing any signs or symptoms. The infection causes a relapse only when certain environmental and nutritional stresses are prevalent. The neutralizing antibodies may therefore contribute to the latency of the disease in humans.

REFERENCES

Arey, L.B., Burrows, W., Greenhill, J.P. and Hewitt, R.M. Eds. 1957 Dorland's Illustrated Medical Dictionary. W.B. Saunders Co., Philadelphia and London. p.75

Barron, A.L. and Riera, M.C. 1969 Studies on hemagglutination by chlamydiae. Proc. Soc. Exptl. Biol. Med. 131:1087

Barron, A.L., Zakay-Rones, Z. and Bernkopf, H. 1965 Hemagglutination of chicken erythrocytes by the agent of psittacosis. Proc. Soc. Exptl. Biol. Med. 119:377

Barwell, C.F. 1952a Some observations on the antigenic structure of psittacosis and lymphogranuloma venereum viruses. I. Preparation and use in complement fixation tests of antiserum from different sources. Brit. J. Exptl. Path. 33:258

Barwell, C.F. 1952b Some observations on the antigenic structure of psittacosis and lymphogranuloma venereum viruses. II. Treatment of virus suspensions by various reagents and the specific activity of acid extract. Brit. J. Exptl. Path. 33:268

Bedson, S.P. 1936 Observations bearing on the antigenic composition of psittacosis virus. Brit. J. Exptl. Path. 17:109

Bedson, S.P. 1938 Study of experimental immunity to the virus of psittacosis in the mouse with special

reference to persistence of infection. Brit. J. Exptl. Path. 19:353

Bedson, S.P. and Western, G.T. 1930 Observations on the virus of psittacosis. Brit. J. Exptl. Path. 11:502

Bell, S.D., Snyder, J.C. and Murray, E.S. 1959 Immunization of mice against toxic doses of homologous elementary bodies of trachoma. Sci. 130:626

Bell, S.D. Jr. and Theobold, B. 1962 Differentiation of trachoma strains on the basis of immunization against toxic death of mice. Ann. N.Y. Acad. Sci. 98:337

Benedict, A.A. and O'Brien, E. 1956 Antigenic studies on the psittacosis lymphogranuloma venereum group of viruses. II. Characterization of complement fixing antigens extracted with sodium lauryl sulphate. J. Immunol. 76:293

Benedict, A.A. and O'Brien, E. 1958 A passive hemagglutination reaction for psittacosis. J. Immunol. 80:84

Bernkopf, H., Nishmi, H., Maythar, B. and Feitelberg, I. 1960 Darkfield agglutination of fluorocarbon-treated trachoma virus by serums of trachoma patients and immunized rabbits. J. Inf. Dis. 106:83

Bietti, G.R., Buogo, A., Chione, M. and Melano, C. 1967 Skin reactions with several trachoma antigens. Amer. J. Ophth. 63:1513

Blyth, W.A., Reeve, P. and Graham, D.M. 1962 The production

of antisera that neutralize inclusion blennorrhea virus. Brit. J. Exptl. Path. 43:340

Caminopetros, J. 1939 Nouvelle recheches sur l'immunite dans la lymphogranulomatose venerienne. Existence d'une reactive particuliere proprea chaque groupe detissus receptifs determinant l'erolution de L'infection. A. Immuntätsforsch 96:217

Collier, L.H. 1966 The present status of Trachoma Vaccination Studies. Bull. WHO. 34:233

Collier, L.H. and Blyth, W.A. 1967 Immunogenicity of experimental trachoma vaccines in baboons. III. Experiments with inactivated vaccine. J. Hyg. Cam. 65:97

Collier, L.H., Duke-Elder, S. and Jones, B.R. 1958 Experimental trachoma produced by cultured virus. Brit. J. Opthalmol. 42:705

Collier, L.H. and Smith, A. 1967 Dissemination and immunogenicity of live TRIC agents in baboons after parenteral injection. Amer. J. Ophth. 43:1589

Collins, A.R. and Barron, A.L. 1970 Demonstration of group specific and species specific antigens of chlamydial agents by gel-diffusion. J. Infect. Dis. 121:1

David, J.R., Al-Askari, S., Lawrence, S. and Thomas, L. 1964a Delayed hypersensitivity in vitro. I. The

specificity of inhibition of cell migration by antigens. J. Immunol. 93:264

David, J.R., Al-Askari, S., Lawrence, S. and Thomas, L. 1964b Delayed hypersensitivity in vitro. II. Effect of sensitive cells on normal cells in the presence of antigen. J. Immunol. 93:274

David, J.R., Al-Askari, S., Lawrence, S. and Thomas, L. 1964c Delayed hypersensitivity in vitro. III. The specificity of hapten-protein conjugates in the inhibition of cell migration. J. Immunol. 93:279

David, J.R. 1968 Macrophage migration. Fed. Proc. 27:6

Davis, D.J. 1948 Complement fixation reactions in psittacosis. Arch. Internal Med. 81:623

Elving, P.J., Markowitz, J.M. and Resenthal, I. 1956 Preparation of buffer systems of constant ionic strength. Analytical Chem. 23:1179

Fauve, R., Dekaris, D. and Dragon 1968 Macrophage spreading inhibition in delayed hypersensitivity. Sci. 160:795

Fauve, R.M., Dekaris, D., Dragon, Raynaud, Marcel 1969 Delayed hypersensitivity and inhibition of macrophages spreading in vivo and in vitro studies of tuberculin and streptococcal hypersensitivities in guinea pigs. J. Immunol. 103:1

Gerloff, R.K. and Watson, R.O. 1967 The radioisotope precipitation test for psittacosis group antibody.

Amer. J. Ophthal. 63:1492

Gogolak, F.M. 1953 The histopathology of murine pneumonitis infection and the growth of the virus in the mouse lung. J. Infect. Dis. 92:254

Gogolak, F.M. and Ross, M.R. 1955 The properties and chemical nature of the psittacosis virus hemagglutinin. Virology 1:474

Graber, R.E. and Pomeroy, M.A. 1958 Ornithosis (psittacosis): an epidemiological study of Wisconsin human outbreak transmitted from turkeys. Amer. J. Pub. Health 48:1469

Grayston, J.T., Wang, S.P. and Johnston, P.B. 1960 Etiologic studies of trachoma in Taiwan. Proc. Soc. Exptl. Biol. Med. 103:596

Grayston, J.T., Wang, S.P., Woolridge, M.S., Yang, Y.F. and Johnston, P.B. 1960 Trachoma studies of etiology, laboratory diagnosis and prevention. J. Amer. Med. Assoc. 172:1577

Greaves, A.B., Hilleman, M.R. Taggart, S.R., Bankhead, A.B. and Feld, M. 1957 Chemotherapy in bubonic lympho-granuloma venereum. A clinical and serological evaluation. Bull. WHO. 16:277

Hilleman, M.R. 1945 Immunological studies on the psittacosis lymphogranuloma venereum complement fixing antigens. III. The solubility in ether of an active fraction. J. Immunol. 53:201

Hilleman, M.R., Haig, D.A., Helmhold, R.J. 1951 The indirect CF, Hemagglutination, and Conglutination Complement absorption tests for virus of PLTV group. J. Immunol. 66:115

Irons, J.V., Denley, M.L. and Sullivan, T.D. 1955 Psittacosis in turkeys and fowls as a source of human infections. In psittacosis, diagnosis, epidemiology and control. F.R. Beaudette Ed. pp. 44-65. Rutgers University Press, New Brunswick, N.J.

Jawetz, E. 1964 Agents of trachoma and inclusion conjuctivitis. Ann. Rev. Microbiol. 18:301

Jawetz, E. and Hanna, L. 1960 Trachoma virus isolated in United States. III. Strain specific effects of tetracycline and penicillin. Proc. Soc. Exptl. Biol. Med. 105:320

Jenkins, H.M., Ross, M.R. and Moulder, J.W. 1961 Species specific antigens from cell walls of the agents of meningopneumonitis and feline pneumonitis. J. Immunol. 86:123

Julianelle, L.A. 1939 Antigenicity of the virus of trachoma. Amer. J. Path. 15:279

Kilham, L. 1948 A skin test antigen common to five viruses of psittacosis lymphogranuloma venereum agents. J. Immunol. 60:157

Kim, Y.B., Bradley, S.G. and Watson, D. 1964 Characterization of early 19S and late 7S Immunoglobulin in the mouse. J. Immunol. 93:798

Kittel 1955 Ornithosis in Norddeutschland. Desinfekt. U. Gesundheitsus 47:65

Lippe t, H. and Brand, G. 1955 Die Komplementbindumgreaktion in der Diagnostik der ornithose (Psittakose). Deut. Med. Wochschr. 80:110

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951 Protein measurements with folin phenol reagent. J. Biol. Chem. 193:265

Luna, L.G. 1968 Manual of histologic staining methods of the Armed Forces Institute of Pathology. Amer. Regist. Path. pp.38-39, 119-120.

Matthiesen, M. and Volkert, M. 1956 Serological diagnosis of ornithosis. A serological follow-up of 163 patients. Danish Med. Bull. 3:245

Mayer, M.M., Croft, C.C. and Gray, M.M. 1948 Kinetic studies on immune hemolysis. J. Exptl. Med. 88:427

Meyer, K.F. 1965 Viral and rickettsial infection of man. Editor - Horsefall and Tamm. pp. 1006

Meyer, K.F. and Eddie, B. 1933 Latent psittacosis infections in mice. Proc. Soc. Exptl. Biol. Med. 30:483

Meyer, K.F. and Eddie, B. 1951 Human carrier of the psittacosis virus. J. Inf. Dis. 88:109

Meyer, K.F. and Eddie, B. 1962 Immunity against some bedsonia in man resulting from infection and in animals from infection or vaccination. Ann. N.Y. Acad. Sci. 98:288

Moulder, J.W., Ruda, J. and Greenland, R.M. 1958 The effect of passage with chloramphenical upon the behaviour of penicillin resistant feline pneumonitis virus during subsequent passage with penicillin. J. Infect. Dis. 102:186

Nicks, R., Sayed, H. and Wilt, J.C. 1970 Studies on delayed sensitivity to chlamydial agents. Presented to the Can. Soc. of Microbiol. June 1970, Halifax, Canada

Ormsbee, R.A., Peacock, M., Tallent, G. and Munoz, J.J. 1968 An analysis of the immune response to Rickettsial antigens in the guinea pig. Acta Virol. 12:78

Osler, A.G., Strauss, J.H. and Mayer, M.M. 1952 Diagnostic complement fixation. I. A method. Amer. J. Syph. Gonor. and Ven. Dis. 36:140

Reeves, P. and Graham, D.M. 1962 A neutralization test for trachoma and inclusion blennorrhea virus grown in HeLa cell culture. J. Gen. Microbiol. 27:177

Rivers, T.M. and Berry, G.P. 1931 Psittacosis Experimentally induced infections in mice. J. Exptl. Med. 54:105

Rivers, T.M., Berry, G.P. and Rhoads, C.P. 1930 Psittacosis observation concerning the experimental disease in

parrots, mice, rabbits, guinea pigs and monkeys. J. Amer. Med. Assoc. 95:579

Rivers, T.M. and Schwentker, F.F. 1934 Vaccination of monkey and laboratory workers against psittacosis. J. Exptl. Med. 60:211

Ross, M.R. and Borman, E.K. 1962 Direct and indirect fluorescent antibody technique for the psittacosis lymphogranuloma venereum trachoma group of agents. J. Bact. 85:851

Ross, M.R. and Gogolak, F.M. 1957 The antigenic structure of psittacosis and feline pneumonitis viruses. I. Isolation of CF antigens with group and species specificity. Virology 3:343

Ross, M.R. and Gogolak, F.M. 1957 The antigenic structure of psittacosis and feline pneumonitis viruses. II. Chemical nature of the alkaline soluble antigens. Virology 3:365

Rudd, G.V. and Burnett, F.M. 1941 Intranasal infection of mice with the virus of psittacosis. Australian J. Exptl. Biol. and Med. Sc. 19:33

St. John, E. and Gordon, F.B. 1947 Studies on the immunological relationship of the psittacosis lymphogranuloma venereum group of viruses. J. Infect. Dis. 80:297

Sayed, H., Nicks, R. and Wilt, J.C. 1971 Preparation and characteristics of LGV antigens involved in cell-mediated immunity. Submitted for publication.

Sayed, H. and Wilt, J.C. 1971 Elevation of IgA and other changes in sera with antibodies to chlamydiae. Can. J. Pub. Health 62:245

sell, S. and Asofsky, R. 1968 Lymphocytes and immunoglobulins. Prog. in Allergy 12:151

Smadel, J.E., Wall, M.J. and Gregg, A. 1943 An outbreak of psittacosis in pigeons involving the production of inclusion bodies and transfer of the disease to man. J. Exptl. Med. 78:189

Snyder, J.L., Bell, S.D., Murray, E.S. 1967 Attempt to immunize a volunteer (with formalin inactivated virus) against experimental trachoma induced by Saudi Arabien strain 2. Ann. N.Y. Acad. 98:368

Stavitsky, A.B. 1954 Micromethods for the study of proteins and antibodies. I. Procedure and general application of hemagglutination and hemagglutination inhibition reactions with tannic acid and protein treated red blood cells. J. Immunol. 72:360

Takatsy, G. 1950 A new method for the preparation of Serial dilutions in quick and accurate way. Kiserletes Orrostudomany 2:393

Tang, F.F., Chang, H.L., Huang, Y.T. and Wang, K.C. 1957 Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryo. Chinen Med. J. 75:429

Vedros, N.A. 1967 Species specific antigens from trachoma and inclusion conjuctivitis (chlamydial) agents. J. Immunol. 99:1183

Wagner, J.C. Golub, O.J. and Andrew, V.W. 1946 Psittacosis vaccines prepared from chick embryo tissues. J. Immunol. 54:35

Wagner, J.C., Golub, O.J. and Andrew, V.W. 1949 Cross immunity studies between virus strains in the psittacosis lymphogranuloma venereum group. J. Inf. Dis. 84:41

Wang, S.D., Grayston, J.T. and Alexander, E.R. 1967 Trachoma vaccine studies in monkeys. Amer. J. Ophthal. 63:1615

Wang, S., Kenny, G.E. and Grayston, J.T. 1967 Characterization of trachoma antigens protective against mouse toxicity. Amer. J. Ophthal. 63: Suppl. 1454

Wassen, E. 1935 Studies of lymphogranuloma inguinale from etiological and immunological points of view. Acute Pathol. Microbiol. Scand. 23:1

Woolridge, R.L. and Grayston, J.T. 1962 Further studies with a CF test for trachoma. Ann. N.Y. Acad. Sci. 98:314

Woolridge, R.L., Grayston, J.T., Cheng, I.H., Yang, C.Y. and Neave, C. 1967 Field trial of a monovalent and of a bivalent mineral oil adjuvant trachoma vaccine in Taiwan school children. Amer. J. Ophthal. 63:1639

Wyman, H., Rigby, C. and Wilt, J.C. 1969 Significance of antibody to the psittacosis - LGV group antigen. Can. J. Microbiol. 15:1173

Yanamura, H.Y. and Meyer, K.F. 1942 Active immunity to microbacteria multiforme in the mouse. J. Immunol. 44:195