# Immunological Studies on *Bacterium tularense*

by

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## Introduction

A review of the numerous immunological studies made on tularemia by many investigators reveals the existence of several problems which have defied solution. Perhaps one of the most basic of these is the mechanism of immunity to tularemia. The view that immunity to tularemia is best acquired through infection, either acute or sub-acute, seems justifiable. Vaccines have been prepared in many ways, from many strains of Bacterium tularense which successfully protect the recipient from clinical infections following exposures. These vaccines have not conferred a solid immunity in exposed laboratory workers or experimental animals, such as the rabbit and guinea pig. In the highly susceptible white mouse vaccines induce little or no immunity (1,2), and have failed to compare with the immunity derived from a sub-lethal injection of living Bact. tularense (3). The problem of this difference in immunogenicity remains unsolved. The same exigency exists in the study of other infections. However, remarkable progress has been made in recent years in such infections as those produced by the anthrax bacillus (4,5,6) and the plague bacillus (7).

Watson et al, (4,6) have reported the separation of two significant components from the inflamatory exudate produced at the site of inoculation in animals with Bacillus

anthracis. The polypeptide component contained large amounts of d(-) glutamic acid and produced a histologic reaction similar to that seen in infection. This component also possessed the ability to increase clotting time with purified fibrinogen. The biological activities of this component could not be demonstrated with glutamyl polypeptide obtained from B. subtilis, nor from the anthrax bacillus when grown in vitro. The second component isolated from oedema fluid was a protein which was capable of stimulating solid immunity in the rabbit but was much less effective in protecting mice, hamsters and guinea pigs. Gladstone (5) has reported an antigen having similar properties to those present in anthrax oedema fluid. This antigen was produced in vitro in filtrates of cultures of B. anthracis in plasma or serum of various animals. This antigen protected rabbits, sheep, and monkeys against 100 or more lethal doses of spores. He found that sera from rabbits and sheep hyperimmunized with the antigen contained no protective antibodies, but further immunization with living virulent bacilli produced effective protective sera. These protective antibodies were not adsorbed with the antigen from culture filtrates or with living bacilli. Watson et al (4) were not able to adsorb the protective antibody with oedema fluid produced by virulent strains. The failure to adsorb the antibodies may indicate an undetected antigen produced by virulent cells in the host.

If such an antigen exists, it must be destroyed by processes involved in extraction, since it cannot be adsorbed by oedema fluid produced by virulent strains.

Serological tests have often failed to reveal an antigenic difference between strains and it has been necessary to detect such differences by chemical means. Although it had been demonstrated that the plague bacillus contained an "envelope" and a somatic antigen, Baker, et al (7) were able to determine the true antigenic structure of this organism and to isolate a crystalline antigen. These workers found that by extracting acetone-killed virulent plague bacilli with neutral salt they were able to obtain a water-soluble and a water-insoluble antigenic component. The water soluble fraction which is highly toxic and immunogenic for mice and rats contained at least 3 antigenic components. The first two were a carbohydrate-protein and a carbohydrate-free protein. These were similar immunogenically in mice but not in guinea pigs. They both produced potent antisera and adsorbed all of the antibody. The third component was a soluble toxic fraction. This component, when separated by adsorption with the carbohydrate-protein or the carbohydrate-free protein was capable of producing antiserum which neutralized the plague toxin. The water-insoluble fraction, containing a phenol-soluble and a phenol-insoluble fraction, contained the antigen protecting guinea pigs. It is interesting to note that the water-soluble

fraction is produced by all virulent strains and by very few avirulent strains. The avirulent strains produce only traces of this fraction. This fraction, which represents the "envelope" antigen shows great host specificity. It is immunogenic for mice, rats, and monkeys, while the strains devoid of "envelope" are immunogenic for guinea pigs but not mice.

The two examples cited are typical of the progress that is being made in the solution of the immunological problems presented in various infections. That the immunity mechanisms involved in plague or anthrax are similar to those in tularemia is not implicit but some comparisons may be made. A host specificity is shown in tularemia in the response of various species to this infection. The rat which possesses an innate natural resistance is easily immunized by the introduction of killed vaccines. The white mouse may be immunized with difficulty and only to a slight degree by killed vaccines but solidly immunized by living cells (3). The rabbit and guinea pig are poorly protected by either viable or killed cells. As it will be shown, however, the sera from these species are equally protective for the white mouse. In contrast to the results obtained with the plague bacillus, the protective substance from immune serum may be adsorbed by living or killed Bact. tularense cells. In tularemia there is no relation between the immunogenic capacity of a strain and its virulence (3). The antigenic

structure of Bact. tularense is not as clearly defined as with the case of the plague or anthrax bacillus.

There has been a need for a convenient and reliable test in the immunological studies on tularemia. Several workers in this field have employed the phenomenon of passive immunization in an attempt to study immunity mechanisms involved. Foshay, et al, have studied the protective capacity of human, goat, and horse sera on rats and have correlated this with the precipitin content of the immune serum (8). Francis and Felton have employed the sera of rabbit, horse, sheep and man in protection tests on the mouse (9). Larson has observed the effect of human serum on the protection of the rat (10). The experimental evidence accumulated in these studies usually showed a prolongation in survival time and a decrease in percentage mortality against challenge with the highly virulent Schu strain. It was hoped that by using passive immunization in the white mouse with a challenge strain of lowered virulence some knowledge of the factors involved in the immunity mechanisms concerned in tularemia might be gained.

After some preliminary investigation it was found that immune serum contained the capacity to protect a normal mouse against a lethal dose of a strain of Bact. tularense of moderate virulence. The protective substance found in immune serum appears to be only a part of the complete protection produced in the immunity process. Since this

protective antibody which is demonstrable in the circulating blood of the immune mouse, is elicited by either viable or killed cells, it is apparent that the antibody is only one of the several which may be produced. Since the immune mouse is refractory to the introduction of a large number of cells of the Schu strain but is able passively to transfer to a normal mouse only a small part of this immunity, the question of an additional unknown major antibody is brought forth. Experimental findings in this laboratory have indicated that viable organisms are essential for the production of the immune state (3). The possibility then exists that a complete refractory state is brought forth by some tissue change produced during the course of multiplication of the living organisms in the host during the immunizing process. Since the protective antibody reported in this study is protective against the strains possessing low virulence and the capacity to kill rapidly, which are indicative of a toxemia, the question arises whether this antibody is anti-toxic in nature.

In this study the mouse protection test provided a means of investigating the protective capacity of sera obtained from the vaccinated or recovered animal. A comparison of the protective capacity of sera from species which vary in immune response was possible. The development and persistance of the protective antibody could be made and

a study of its relation to agglutinins was possible. Through adsorption tests and treatment of the antigen some of the factors involved in immunity to tularemia could be tested. Evidence accumulated from the protection tests pointed toward the presence of a toxin in some strains of Bact. tularense. The toxicity of certain strains may be a criterion of variation in antigenic structure in this bacterium. Because of the difference in immunogenicity of various strains in the white mouse (3) some suggestion of a difference in antigenic structure has already been made. This study again suggests this possibility on the basis of toxicity of the different strains.

## Methods and Materials

Mice. All of the mice employed in these investigations were obtained from the Maple Grove Rabbitry, Springfield, Mo. They represented a strain of white mice of known susceptibility to tularemia. They were selected without regard to sex and their weight approximated 20 g.

Strains of Bacterium tularense. The Jap strain of Bact. tularense was employed as the antigen for the production of immune mouse serum. This strain is of moderate virulence with an approximate $LD_{50}$ of $10^{-3.5}$ for the white mouse when inoculated intraperitoneally or subcutaneously. The Jap strain was isolated in Japan in 1926 from a human lymph node.

Mouse protection test. The immune serum which contained the protective capacity was obtained in the following manner: A saline suspension of the Jap strain of Bact. tularense was prepared from a 24 hour culture grown on glucose cysteine blood agar (GCBA) slant to give 24 per cent light transmission in the Coleman spectrophotometer at a wave length of 600. This standard suspension contains approximately 1 to 4 x $10^9$ organisms per ml. Decimal dilutions of this standard suspension were prepared in 0.85 per cent sodium chloride. An immunizing dose of 0.5 ml of a $10^{-5}$ saline suspension was injected intraperitoneally into the desired number of susceptible white mice. This inoculum

contained approximately 50,000 organisms. This group of immunized mice was bled from the heart on the 5th, 6th, or 7th post-inoculation day. The blood was pooled, refrigerated, and the serum separated. To demonstrate the protective capacity of the serum, equal quantities of this serum and a $10^{-1}$ dilution of a standard suspension of the Jap strain were inoculated into a group of normal white mice. The inocula usually consisted of 0.5 ml of immune serum plus 0.5 ml of a $10^{-1}$ suspension of Bact. tularense, containing approximately 1 x $10^{8}$ cells. No incubation period was observed before inoculation. The infected mice were observed for a period of 10 days. The day of death and mortality percentage were recorded. Surviving mice were challenged with 1000 $LD_{50}$ of the highly virulent Schu strain of Bact. tularense.

A mouse protection index (MPI) was calculated in a manner similar to that of Meyer and Foster (11). Since Meyer found in his work on the plague bacillus that little significance could be attached to either the percentages of mortality or the lengths of survival time, he considered the two factors together. He designated the percentage mortality divided by the average time of death as the "mouse protection index." A small index number represents greater protection than a large number. Theoretically, an MPI of 100 represents no protection, while an MPI of zero represents complete protection. If 100 per cent of the mice died in 1 day, the

MPI was 100 and the serum gave no protection. By using 100 $LD_{50}$ of the Jap strain as the challenging inoculum with normal mouse serum, an MPI of 50 to 100 was usually obtained for the controls. Because of slight individual variation in susceptibilty found in the white mouse and variation in the killing time of the different strains of Bact. tularense, all experiments were carried out with controls of normal mouse serum. In this manner, it was possible to calculate an MPI for the immune serum employed and an MPI for the normal serum employed. For further verification of the protective capacity of serum a "protection ratio" (PR) was calculated. This is a direct comparison of the MPI of the normal serum to the MPI of the immune serum. A PR approaching 100 indicates nearly complete protection. In the event that all protected mice survived, the MPI is not computable. Since the challenge dose used throughout these experiments was greater than one permitting complete protection, this mathematical impossibility occurred but rarely. In the event that complete protection did occur, the result was simply expressed as "complete," rather than by a numerical expression.

Agglutination tests. A formalinized suspension of the 38 strain of Bact. tularense was used as the antigen. The turbidity of the suspension was prepared to match a standard suspension as defined above. Two-fold saline dilutions of serum were made employing 0.2 ml transfers. The

suspensions were refrigerated overnight at 4° C and read on the following day.

Adsorption tests. Immune serum was adsorbed by using a very heavy suspension of cells, living or killed as stated in the experiments, in a minimum volume of saline. The serum-cell suspension was refrigerated overnight at 4° C. The serum was cleared of cells by centrifugation and a rapid slide agglutination test was performed to check for complete adsorption. When viable cells were employed a sample of serum was plated to check sterility.

Spleen extracts. The spleens were aseptically removed from the mice as soon after death as possible. They were immediately frozen on a dry-ice-acetone preparation and kept frozen until use. The spleens were weighed and a sufficient amount of saline added to give a final suspension of 0.25 gm spleen per ml saline. The spleen-saline mixture was homogenized in a Waring blender for approximately 2 minutes. The homogenate was centrifuged at 4° C at 2500 rpm for ½ hour. The supernate was filtered through a Seitz filter. In this manner the possibility of residual organisms was excluded.

Sera tested. In the routine mouse protection test the serum employed was obtained from infected mice as stated above. In the experiments in which sera of the rat, rabbit, guinea pig and human were used, the data pertaining to these sera are presented in Table 15.

## Experimental Results

Development of the protective substance in immune serum.

In order to demonstrate the appearance of the protective substance in immune serum, a group of 100 white mice were inoculated with approximately 51,000 organisms of the Jap strain of Bact. tularense. Ten of these mice were bled daily and the pooled serum or whole blood was added in equal volume to a lethal suspension of Jap organisms. This inoculum was injected intraperitoneally into 6 normal mice which were observed for a period of 10 days. The results presented in Table 1 and Figure 1 indicate that there is apparent on the third to fourth day some evidence of a protective capacity in immune serum and whole blood. The irregularity of the amount of protection obtained probably is a reflection of the individual response of the mouse. I is apparent that the protection which becomes evident after the third day has not decreased noticeably at the end of 10 days.

Duration of protection.

A group of normal mice was injected intraperitoneally with approximately 40,000 Jap organisms. Two weeks following this inoculation, 14 mice were bled for serum. The serum was added to an equal volume of Jap organisms and inoculated into a group of 10 normal mice. This procedure was repeated at weekly intervals for 7 weeks. The results,

presented in Table 2 and Figure 2, show that the protective capacity of the serum remained for at least 7 weeks. Again, an irregularity in the degree of protection afforded is evident and reflects individual differences in mouse antibody response.

Table I

Development of Protective Substance in Serum and Whole Blood From Infected Mice

| | Test Mice* | | | | | Control Mice** | | | | PR |
|---|---|---|---|---|---|---|---|---|---|---|
| Day of Bleeding | Dead Tested | Per cent Mortality | Av. Day of Death | Titer | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| 1 | 6/6 | 100 | 4.5 | 0 | 22.2 | 6/6 | 100 | 4.1 | 24.3 | 1.09 |
| 2 | 6/6 | 100 | 4.1 | 0 | 24.3 | 6/6 | 100 | 3.6 | 27.7 | 1.14 |
| 3 | 4/6 | 66 | 5.8 | 0 | 11.3 | 6/6 | 100 | 4.6 | 21.7 | 1.91 |
| 4 | | | | 0 | | | | | | |
| 5 | 2/6 | 33 | 5.0 | 0 | 6.6 | 6/6 | 100 | 3.1 | 32.2 | 4.87 |
| 6 | 3/6 | 50 | 5.3 | 1:20 | 9.4 | 6/6 | 100 | 5.0 | 20.0 | 2.12 |
| 7 | 3/6 | 50 | 7.3 | 1:20 | 6.8 | 6/6 | 100 | 3.6 | 27.7 | 4.07 |
| 8 | 2/6 | 33 | 10.0 | 1:80 | 3.3 | 6/6 | 100 | 3.8 | 26.3 | 7.97 |
| 9 | | | | 1:40 | | | | | | |
| 10 | 3/6 | 50 | 8.0 | 1:20 | 6.2 | 6/6 | 100 | 5.6 | 17.8 | 2.87 |

* Test mice received serum from infected mice + 1 x $10^7$ Jap cells.

** Control mice received serum from normal mice + 1 x $10^7$ Jap cells.

Table I (continued)

| | Test Mice# | | | | | Control Mice## | | | | PR |
|---|---|---|---|---|---|---|---|---|---|---|
| Day of Bleeding | Dead Tested | Per cent Mortality | Av. Day of Death | Titer | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| 1 | 6/6 | 100 | 4.6 | 0 | 21.7 | 6/6 | 100 | 5.3 | 18.8 | .86 |
| 2 | 6/6 | 100 | 6.6 | 0 | 15.1 | 5/6 | 83 | 4.4 | 18.8 | 1.24 |
| 3 | 5/6 | 83 | 4.0 | 0 | 20.7 | 5/6 | 83 | 3.4 | 24.4 | 1.13 |
| 4 | 4/6 | 66 | 5.2 | 0 | 12.6 | 6/6 | 100 | 3.1 | 32.2 | 2.55 |
| 5 | 2/6 | 33 | 7.5 | 1:40 | 4.4 | 6/6 | 100 | 4.1 | 24.3 | 5.52 |
| 6 | 1/6 | 16 | 6.0 | 1:40 | 2.6 | 3/6 | 50 | 4.3 | 11.6 | 4.46 |
| 7 | 5/6 | 83 | 5.3 | 1:80 | 15.6 | 6/6 | 100 | 3.1 | 32.2 | 2.06 |
| 8 | 2/6 | 33 | 5.5 | 1:80 | 6.0 | 6/6 | 100 | 3.6 | 27.7 | 4.61 |
| 9 | 2/6 | 33 | 5.5 | 1:40 | 6.0 | 6/6 | 100 | 4.3 | 23.3 | 3.88 |
| 10 | 2/6 | 33 | 5.5 | 1:40 | 6.0 | 6/6 | 100 | 3.3 | 30.3 | 5.05 |

\# Test mice received whole blood from infected mice $\neq 1 \times 10^7$ Jap cells.

\#\# Control mice received whole blood from normal mice $\neq 1 \times 10^7$ Jap cells.

Figure I

## Development of Protective Substance

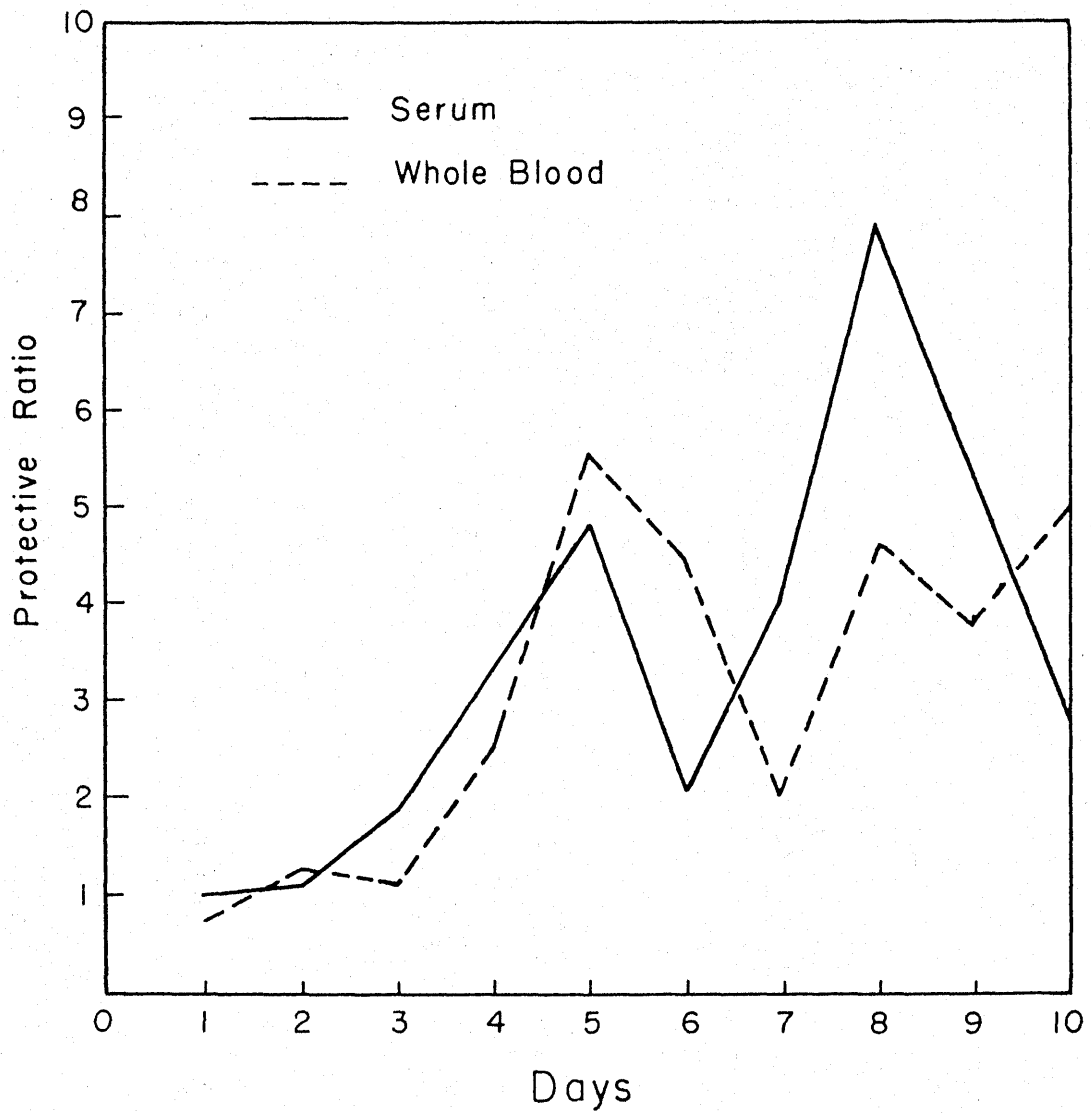


Table 2

Duration of Protective Antibodies in House Serum Following Active Immunisation

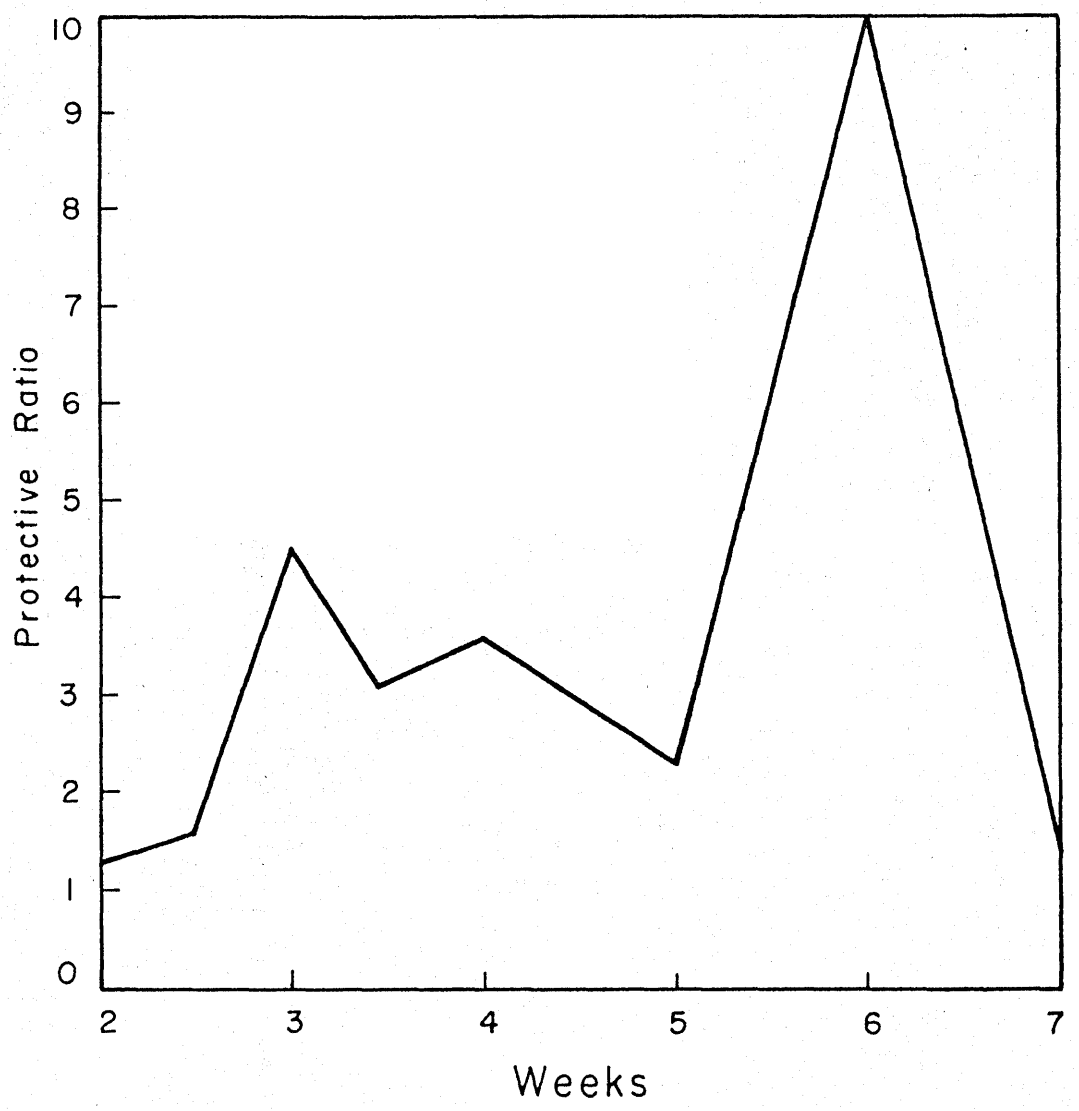
| | Test Mice* | | | | | Control Mice** | | | | PR |
|---|---|---|---|---|---|---|---|---|---|---|
| Inoculum with Jap Serum | Day | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| $1.5 \times 10^3$ | 14 | 7/10 | 70 | 4.7 | 14.8 | 3/3 | 100 | 5.3 | 18.8 | 1.27 |
| $4.0 \times 10^8$ | 21 | 9/10 | 90 | 4.1 | 21.9 | 3/3 | 100 | 1.0 | 100.0 | 4.52 |
| $3.0 \times 10^8$ | 24 | 5/8 | 63 | 5.0 | 12.6 | 3/3 | 100 | 2.5 | 40.0 | 3.17 |
| $4.0 \times 10^8$ | 28 | 10/10 | 100 | 5.8 | 17.2 | 5/5 | 100 | 1.6 | 62.5 | 3.63 |
| $3.0 \times 10^8$ | 35 | 8/8 | 100 | 4.7 | 21.2 | 4/4 | 100 | 2.0 | 50.0 | 2.31 |
| $3.5 \times 10^8$ | 42 | 3/6 | 50 | 8.0 | 6.2 | 3/3 | 100 | 1.6 | 62.5 | 10.08 |
| $2.0 \times 10^8$ | 49 | 3/3 | 100 | 5.3 | 18.8 | 4/4 | 100 | 3.7 | 27.0 | 1.42 |

* The test mice received Jap immune serum + the inoculum indicated.

** The control mice received normal mouse serum + the inoculum indicated.

Figure II

## Duration of Protection



Minimal amount of serum necessary for protection.

A series of protection tests were carried out on various amounts of serum, ranging from 1.5 ml to 0.001 ml. When the limiting amount of serum necessary for protection was determined roughly, smaller increments of sera were employed to define the actual limit of protection. The results are presented in Table 3 and Figure 3. It is seen that 0.01 ml of serum protected mice against the usual challenge dose. The PR obtained with 0.0075 ml serum is just below the limit accepted as evidence of protection. The PR continues to decrease directly with a decrease in the amount of serum employed. No protection was procured with 0.001 ml serum. It may also be seen from Figure 3 that the degree of protection is not greatly augmented by an increase in the amount of serum employed, once a sufficient amount is introduced into the mouse to afford protection.

Table 3

Minimal Amount of Serum Necessary for Protection

| | Test Mice* | | | | Control Mice ** | | | | PR |
|---|---|---|---|---|---|---|---|---|---|
| Amount of serum- ml | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| 1.5000 | 5/6 | 83 | 5.2 | 15.9 | 4/4 | 100 | 2.2 | 45.4 | 2.85 |
| 1.0000 | 3/6 | 50 | 5.3 | 9.4 | 3/4 | 75 | 1.3 | 57.6 | 6.12 |
| 0.8000 | 5/6 | 83 | 5.4 | 15.3 | 4/4 | 100 | 1.5 | 66.6 | 4.35 |
| 0.5000 | 5/6 | 83 | 5.4 | 15.3 | 4/4 | 100 | 1.5 | 66.6 | 4.35 |
| 0.2500 | 6/6 | 100 | 4.3 | 23.2 | 4/4 | 100 | 1.2 | 83.3 | 3.59 |
| 0.1000 | 4/6 | 66 | 4.5 | 14.6 | 4/4 | 100 | 1.5 | 66.6 | 4.56 |
| 0.0100 | 5/6 | 83 | 2.8 | 26.0 | 4/4 | 100 | 1.2 | 83.3 | 3.20 |
| 0.0075 | 6/6 | 100 | 2.3 | 43.4 | 3/3 | 100 | 1.6 | 62.5 | 1.44 |
| 0.0050 | 6/6 | 100 | 2.0 | 50.0 | 3/3 | 100 | 2.0 | 50.0 | 1.00 |
| 0.0025 | 6/6 | 100 | 2.1 | 47.6 | 3/3 | 100 | 2.3 | 43.4 | 0.91 |
| 0.0010 | 6/6 | 100 | 1.5 | 66.6 | 4/4 | 100 | 1.7 | 58.8 | 0.88 |

* Test mice received $1 \times 10^8$ Jap cells + the amount of immune serum indicated.

** Control mice received $1 \times 10^8$ Jap cells + the amount of normal serum indicated.

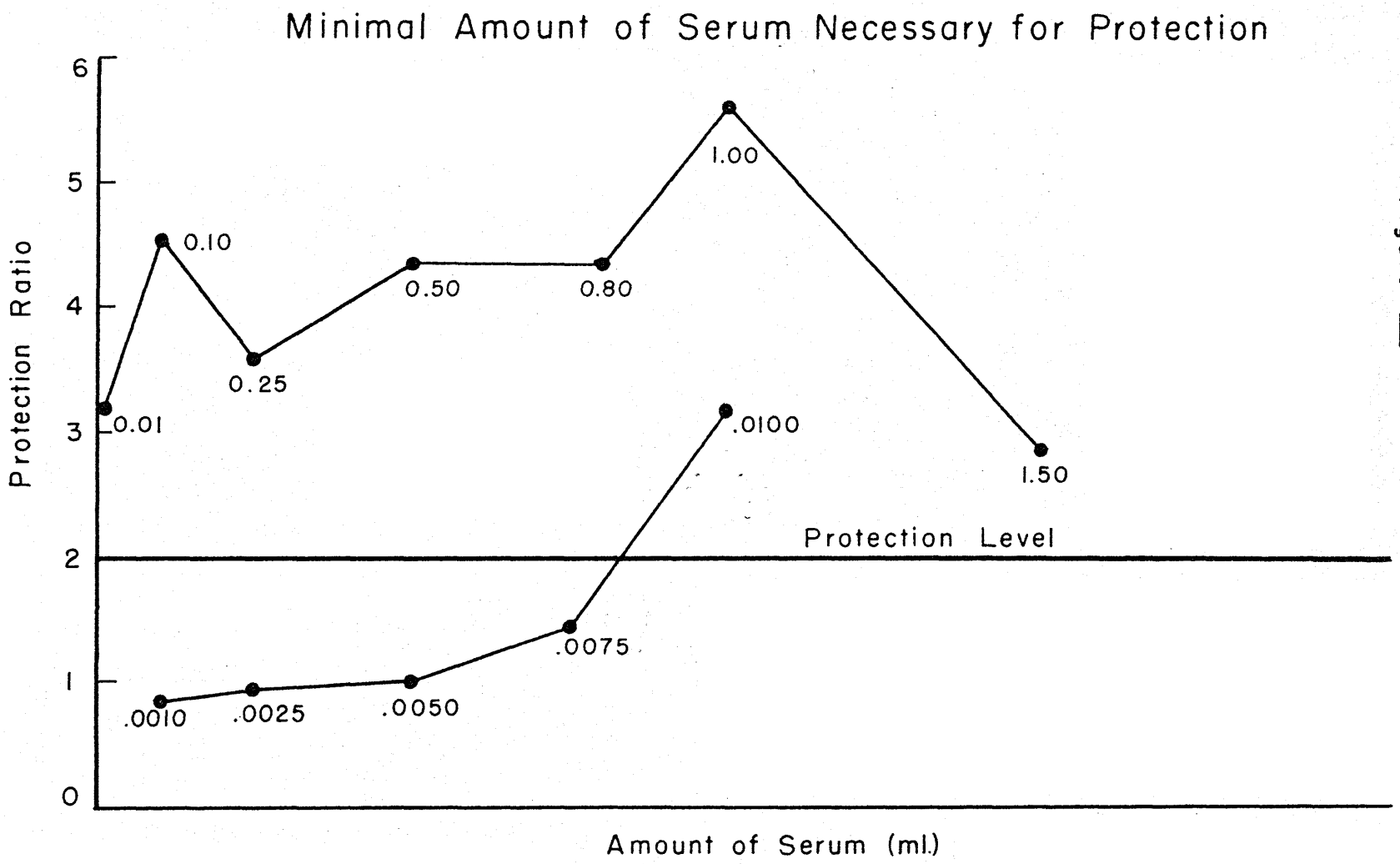

Minimal Amount of Serum Necessary for Protection
Protection Ratio
Amount of Serum (ml.)
Protection Level
0
1
2
3
4
5
6
0.01
0.10
0.25
0.50
0.80
1.00
1.50
.0010
.0025
.0050
.0075
.0100

Figure III

Relation between protection and agglutinin content of immune serum.

A group of 200 white mice were injected with 43,500 Jap cells. Ten mice were sacrificed daily on the second through the twenty-second day following the inoculation. Of these 10 mice, 3 were bled separately to determine the agglutination titer. The excess sera from these 3 mice were added to the pool of serum obtained from the 7 mice. Daily tests for protection were performed by the injection of 6 normal mice with a combination of 0.5 ml of a Jap suspension of approximately 1 x $10^8$ cells plus 0.5 ml immune Jap serum. On the fifteenth post-inoculation day, the remaining 50 mice from the original group were given a "booster dose" of approximately 4 x $10^6$ Jap cells. Daily agglutination tests and protection tests were performed on the sera of these mice for the remaining 7 days. The results of this experiment are presented in Table 4 and Figure 4.

From these results it is apparent that a protective capacity is evident before an agglutination titer is demonstrable. The protective substance is present on the third day and rises sharply on the fourth day. The first evidence of an agglutination titer is seen in 1 out of 3 mice on the fourth day, 2 out of 3 mice on the fifth day, and 3 out of 3 mice on the sixth day. The agglutination titer reached a peak in the 3 mice sampled on the tenth to

thirteenth day. Following the "booster dose," the response in titer was immediate with a peak reached on the day following reinoculation. The protective capacity remained in evidence throughout the daily tests. In response to the "booster dose" the protective capacity increased gradually to reach a peak on the fifth post-reinoculation day. Neither the agglutination titer nor the PR had reached a negative point at the termination of the experiment. The variation found in individual mouse agglutinin response is apparent.

Table 4

Relation of Agglutinins to Protective Substance

| | Test Mice* | | | | | | Control Mice* | | | | PR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | Titers 3 Mice | | | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| 2 | 0 | 0 | 0 | 6/6 | 100 | 1.5 | 66.6 | 3/3 | 100 | 1.6 | 62.5 | 0.93 |
| 3 | 0 | 0 | 0 | 6/6 | 100 | 2.5 | 40.0 | | | | | 1.56 |
| 4 | 0 | 0 | 4 | 3/5 | 60 | 4.6 | 13.0 | | | | | 4.80 |
| 5 | 0 | 8 | 16 | 7/7 | 100 | 4.7 | 21.2 | 4/4 | 100 | 2.2 | 45.4 | 2.14 |
| 6 | 8 | 8 | 32 | 5/6 | 83 | 4.0 | 20.7 | | | | | 2.19 |
| 7 | 0 | 32 | 32 | 5/5 | 100 | 5.6 | 17.8 | 3/3 | 100 | 4.6 | 21.7 | 1.21 |
| 8 | 8 | 16 | 128 | 7/7 | 100 | 4.7 | 21.3 | | | | | 1.01 |
| 9 | 16 | 32 | 64 | 6/6 | 100 | 5.0 | 20.0 | | | | | 1.85 |
| 10 | 128 | 64 | 64 | 3/5 | 60 | 5.6 | 10.7 | 4/4 | 100 | 3.7 | 27.0 | 2.52 |
| 11 | 64 | 32 | 16 | 6/6 | 100 | 4.3 | 23.2 | | | | | 1.16 |
| 12 | 64 | 16 | 0 | 3/7 | 42 | 4.3 | 9.7 | | | | | 2.78 |

* For legend, see text.

Table 4 (continued)

| Day | Titers 3 Mice | | | Test Mice* Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Control Mice* Dead Tested | Per cent Mortality | Av. Day of Death | MPI | PR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | 64 | 32 | 0 | 0/4 | 0 | none | 0 | | | | | Complete |
| 14 | 0 | 0 | 0 | 2/5 | 40 | 4.5 | 8.8 | 3/3 | 100 | 2.3 | 43.4 | 4.96 |
| 15 | 8 | 0 | 0 | 5/7 | 72 | 4.8 | 14.7 | | | | | 2.95 |
| | | | | | | "Booster Dose" | | | | | | |
| 16 | 16 | 32 | 128 | 8/9 | 88 | 5.3 | 16.6 | 4/4 | 100 | 2.7 | 37.0 | 2.22 |
| 17 | 64 | 32 | 16 | 5/8 | 62 | 5.0 | 12.4 | | | | | 2.98 |
| 18 | 0 | 0 | 0 | 4/7 | 57 | 5.5 | 10.3 | | | | | 3.53 |
| 19 | 32 | 16 | 8 | 5/7 | 71 | 5.4 | 13.1 | 4/4 | 100 | 1.0 | 100.0 | 7.63 |
| 20 | 16 | 16 | 4 | 4/8 | 50 | 5.2 | 9.6 | | | | | 10.41 |
| 21 | 16 | 8 | 4 | 5/6 | 83 | 5.2 | 15.7 | | | | | 6.36 |
| 22 | 16 | 16 | 16 | 4/5 | 80 | 6.0 | 15.0 | 3/3 | 100 | 5.6 | 17.8 | 1.18 |

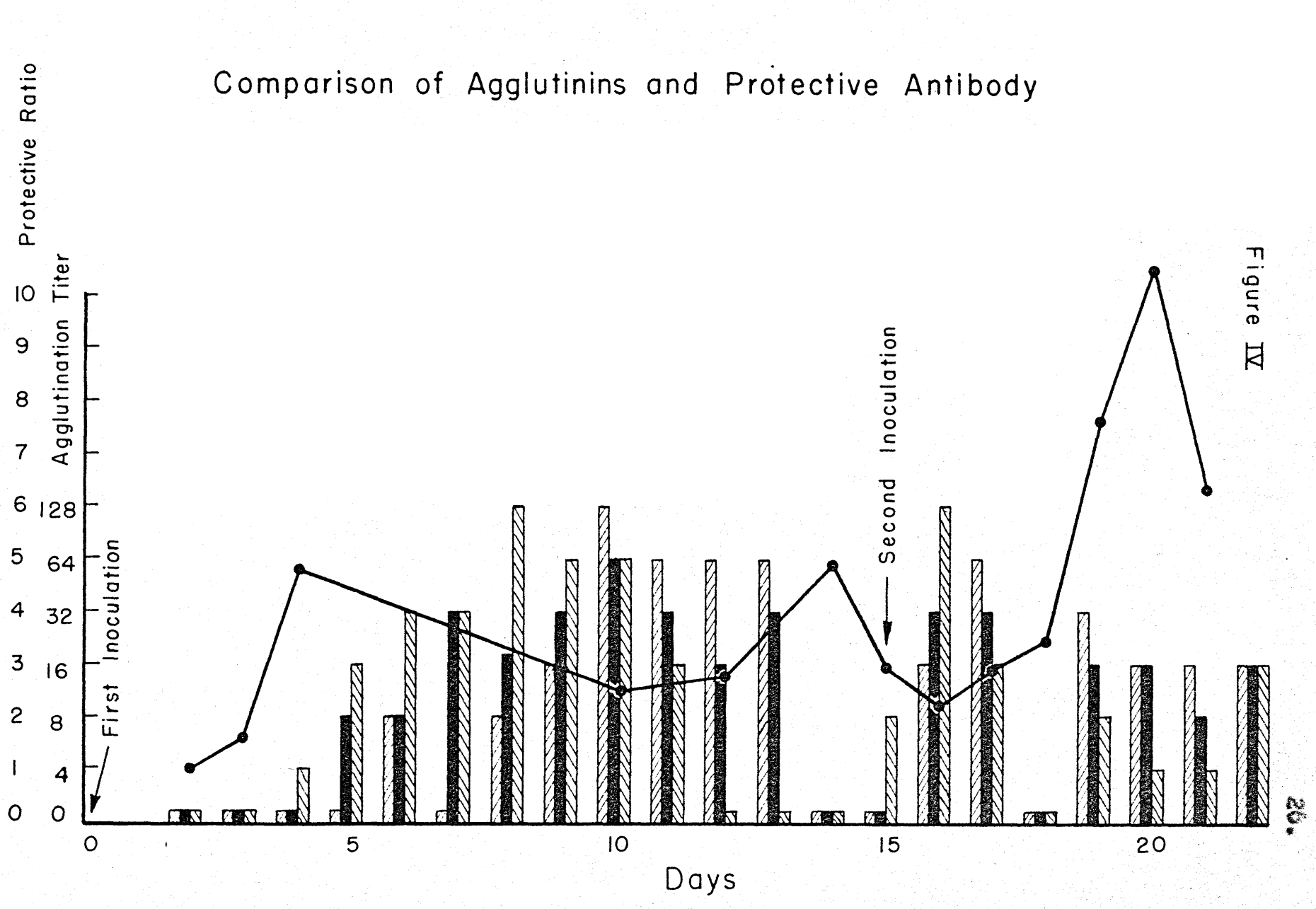

Comparison of Agglutinins and Protective Antibody
Protective Ratio
Agglutination Titer
0
1
2
3
4
5
6
7
8
9
10
0
4
8
16
32
64
128
First Inoculation
Second Inoculation
0
5
10
15
20
Days

Figure IX

Development of active immunity in the passively protected mouse.

A group of 75 normal mice was inoculated intraperitoneally with a mixture of equal quantities of immune serum and approximately 100 $LD_{50}$ doses of the Jap strain of Bact. tularense. Each mouse received 0.5 ml. of immune serum and 0.5 ml of a suspension of approximately 1 x $10^8$ Jap cells. Each day a small percentage of these mice were selected for injection of 1000 $LD_{50}$ doses of the Schu strain. The challenged mice were observed for a period of 14 days. The daily challenge doses were injected into 3 normal mice for controls. The results of this experiment, recorded in Table 5, show that active immunity is present at least by the fifth post-inoculation day.

Heat stability of the protective substance.

Immune serum was collected from mice which had survived an injection of the Jap strain of Bact. tularense. This serum was divided into 4 equal parts. One pool of serum was untreated. The other 3 pools were heated for one-half hour at 56°, 60°, and 65° C, respectively. The 4 pools of sera were then added in equal portions to a saline suspension containing 3.4 x $10^8$ Jap cells. The results of the protection tests carried out on the heated and unheated sera are presented in Table 6. No significant loss in protection by heat treatment is apparent from these results.

Table 5

Development of Active Immunity in the Passively Protected Mouse

| | Test Mice* | | | | Control Mice** | | | | PR |
|---|---|---|---|---|---|---|---|---|---|
| Challenge Day | Dead Tested | Per cent Mortality | Av. Day of Death | MFI | Dead Tested | Per cent Mortality | Av. Day of Death | MFI | |
| 1 | 5/5 | 100 | 3.2 | 31.2 | 3/3 | 100 | 4.0 | 25.0 | 0.83 |
| 2 | 4/5 | 80 | 4.0 | 20.0 | 3/3 | 100 | 3.6 | 27.7 | 1.38 |
| 3 | 9/10 | 90 | 2.6 | 34.6 | 3/3 | 100 | 2.3 | 43.4 | 1.25 |
| 4 | 9/10 | 90 | 3.2 | 28.1 | 3/3 | 100 | 3.6 | 27.7 | 0.99 |
| 5 | 4/5 | 80 | 7.0 | 11.4 | 3/3 | 100 | 4.0 | 25.0 | 2.28 |
| 11 | 0/4 | 0 | none | 0 | 3/3 | 100 | 4.0 | 25.0 | complete |

* Test mice had received immune serum + $1 \times 10^8$ Jap cells on day indicated previous to challenge with 1000 $LD_{50}$ Schu cells.

** Control mice were normal mice injected with 1000 $LD_{50}$ Schu cells.

Table 6

Protective Capacity of Heated Immune Serum

| | Test Mice* | | | | Control Mice** | | | | PR |
|---|---|---|---|---|---|---|---|---|---|
| Treatment of Serum | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| Unheated | 13/19 | 68 | 6.5 | 10.4 | 6/6 | 100 | 2.8 | 35.7 | 3.43 |
| 56° ½ hour | 13/20 | 60 | 5.6 | 10.7 | | | | | 3.33 |
| 60° ½ hour | 13/20 | 65 | 6.0 | 10.8 | | | | | 3.30 |
| 65° ½ hour | 13/20 | 65 | 5.4 | 12.0 | | | | | 2.97 |

* Test mice received the treated serum as indicated + $3.4 \times 10^8$ Jap cells.

** Control mice received unheated serum + $3.4 \times 10^8$ Jap cells.

Specificity of the protective substance.

The protection of Jap serum against a heterologous strain was tested by the usual mouse protection test. The immune Jap serum was added in equal volume to a suspension of a heterologous strain composed of amounts varying from 1-1000 $LD_{50}$. Table 7 presents the results obtained for protection of Jap serum against the various strains tested. Table 8 shows the $LD_{50}$ of the strains used together with the number of $LD_{50}$ tested and the results obtained. It may be noted that Jap immune serum protects well against the Jap, Russ, Church, HD and Schad strains. It protects less well against the Coll strain. The results obtained against the Ince strain were variable and could not be repeated. Complete protection was obtained in 2 experiments with a challenge dose of approximately $1.4 \times 10^3$ Ince cells while in another experiment the PR was 2.34 against a challenge dose of $1.1 \times 10^3$ Ince cells. Jap immune serum did not protect against the Schu, Bish or RI strains. The data presented in Table 9 were taken from an experiment carried on in this laboratory in work not dealing with the mouse protection test. The data is presented to substantiate the finding that the strains Schad, HD, Stoll and Depue are more toxic than the Coll or Schu strains. This is indicated by the later average day of death of mice challenged with the Coll or Schu strains. The evidence indicates that the mice previously immunized with

low virulent strains retained some immunity against the challenge doses as seen by the percentages of mortality. However, the Schu strain produced a slower death even when challenged with $2.4 \times 10^8$ Schu cells which represent 4 million $LD_{50}$. The average day of death with the Schad challenge was less than 2 with the immunized mice and 1 with the control mice. The Schad challenge consisted of approximately 10 $LD_{50}$.

Table 7

Specificity of the Protective Substance

| Challenge Dose and Strain | Test Mice* Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Control Mice** Dead Tested | Per cent Mortality | Av. Day of Death | MPI | PR |
|---|---|---|---|---|---|---|---|---|---|
| Schu $3.1 \times 10^3$ | 5/5 | 100 | 3.8 | 26.3 | 5/5 | 100 | 3.8 | 26.3 | 1.00 |
| Schu $2.5 \times 10^2$ | 11/11 | 100 | 4.5 | 22.2 | 9/9 | 100 | 4.1 | 24.3 | 1.09 |
| Schu $4.0 \times 10^1$ | 30/30 | 100 | 5.0 | 20.0 | 12/12 | 100 | 4.6 | 21.7 | 1.08 |
| Schu 3.1 | 9/15 | 60 | 5.3 | 11.3 | 4/5 | 80 | 4.7 | 21.0 | 1.87 |
| FI $1.7 \times 10^7$ | 26/27 | 96 | 5.7 | 16.8 | 10/10 | 100 | 3.9 | 25.6 | 1.52 |
| FI $1.6 \times 10^6$ | 13/15 | 86 | 6.1 | 14.0 | 7/7 | 100 | 4.4 | 22.7 | 1.62 |
| Ince $1.4 \times 10^4$ | 12/15 | 80 | 6.6 | 12.1 | 8/8 | 100 | 6.6 | 12.1 | 1.00 |
| Ince $1.1 \times 10^3$ | 3/12 | 25 | 7.0 | 3.5 | 2/3 | 66 | 8.0 | 8.2 | 2.34 |
| Ince $1.4 \times 10^3$ | 0/11 | 0 | none | 0.0 | 6/8 | 75 | 5.1 | 14.7 | complete |
| Russ $3.3 \times 10^9$ | 3/6 | 50 | 7.0 | 7.1 | 4/4 | 100 | 1.0 | 100.0 | 14.08 |

* Test mice received Jap immune serum ≠ the strain and dose indicated.

** Control mice received normal mouse serum ≠ the strain and dose indicated.

Table 7 (continued)

| Challenge Dose and Strain | Test Mice: Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Control Mice: Dead Tested | Per cent Mortality | Av. Day of Death | MPI | PR |
|---|---|---|---|---|---|---|---|---|---|
| Jap $2.6 \times 10^8$ | 18/36 | 50 | 5.8 | 0.86 | 15/15 | 100 | 1.3 | 76.1 | 88.48 |
| Coll $1.9 \times 10^9$ | 3/6 | 50 | 4.3 | 11.6 | 4/4 | 100 | 3.0 | 33.3 | 2.87 |
| Bish $2.0 \times 10^5$ | 5/6 | 83 | 5.3 | 15.9 | 4/4 | 100 | 4.7 | 21.2 | 1.33 |
| Bish $2.0 \times 10^4$ | 6/6 | 100 | 7.8 | 12.8 | 4/4 | 100 | 5.0 | 20.0 | 1.56 |
| Church $1.3 \times 10^8$ | 0/6 | 0 | none | 0 | 4/4 | 100 | 3.7 | 27.0 | complete |
| Church $1.3 \times 10^7$ | 0/6 | 0 | none | 0 | 3/4 | 75 | 4.7 | 15.9 | complete |
| MD $1.0 \times 10^9$ | 0/6 | 0 | none | 0 | 2/4 | 50 | 1.0 | 50.0 | complete |
| Schad $6.3 \times 10^9$ | 0.6 | 0 | none | 0 | 3/4 | 75 | 1.3 | 57.6 | complete |

Table 8

Comparison of Protection Ratios on the Basis of $LD_{50}$

| Strain | $LD_{50}$ | $LD_{50}$ Dose | Av. Day of Death in Controls | Av. Day of Death in Protected Mice | PR |
|---|---|---|---|---|---|
| Schu | $10^{-9.3}$ | 1000 | 3.8 | 3.8 | 1.00 |
| | | 100 | 4.0-4.2 | 4.4-4.5 | 1.07-1.10 |
| | | 10 | 4.2-4.5 | 4.7-5.5 | 0.90-1.22 |
| | | 1 | 4.7 | 5.3 | 1.85 |
| Ince | $10^{-8.7}$ | 1000 | 6.2-7.0 | 5.2-8.0 | 0.84-1.96 |
| | | 100 | 4.3-8.0 | 7.0-none | 2.34-complete |
| Bish | $10^{-6.5}$ | 100 | 4.7 | 5.3 | 1.33 |
| | | 10 | 5.0 | 7.8 | 1.56 |
| EI | $10^{-5.9}$ | 1000 | 3.6-4.2 | 4.6-6.4 | 1.27-1.46 |
| | | 100 | 4.3-4.5 | 6.0-6.2 | 1.50-1.66 |
| Jap | $10^{-3.5}$ | 1000 | 1.0 | 1.2 | 1.20 |
| | | 100 | 1.0-2.0 | 4.5-7.5 | 4.11-29.40 |
| | | 10 | 3.0 | 8.0 | 11.00 |
| Church | $10^{-3.3}$ | 100 | 3.7 | none | complete |
| | | 10 | 4.7 | none | complete |
| Russ | $10^{-1.5}$ | 10 | 1.0 | 7.0 | 14.08 |
| HD | $10^{-1.5}$ | 10 | 1.0 | none | complete |
| Coll | $10^{-1.4}$ | 10 | 3.0 | 4.3 | 2.87 |
| Schad | $10^{-0.5}$ | 1 | 1.3 | none | complete |

Table 9

Comparison of Average Days of Death in Mice Partially Immunized with Strains of Bact. tularense

| Challenge Strain and Dose | $LD_{50}$ Doses | Mice Tested* | Dead Tested | Per cent Mortality | Av. Day of Death |
|---|---|---|---|---|---|
| Schad $5.4 \times 10^9$ | 10 | Normal | 7/10 | 70 | 1.00 |
| | | 37 days | 11/13 | 85 | 1.18 |
| | | 34 days | 11/12 | 92 | 1.45 |
| | | 24 days | 9/13 | 69 | 1.11 |
| HD $4.8 \times 10^9$ | 200 | Normal | 3/3 | 100 | 1.33 |
| | | 37 days | 9/9 | 100 | 1.22 |
| | | 34 days | 7/9 | 78 | 1.14 |
| Stoll $7.7 \times 10^9$ | 10 | Normal | 8/10 | 80 | 1.62 |
| | | 37 days | 10/14 | 71 | 1.80 |
| | | 34 days | 5/9 | 56 | 1.40 |
| Dapue $1.2 \times 10^9$ | 2 | Normal | 2/5 | 40 | 1.00 |
| | | 37 days | 6/12 | 50 | 1.16 |
| | | 34 days | 5/8 | 62 | 1.20 |
| Coll $1.5 \times 10^9$ | 20 | Normal | 10/10 | 100 | 2.30 |
| | | 37 days | 10/13 | 77 | 1.40 |
| | | 34 days | 12/17 | 71 | 2.00 |
| | | 24 days | 10/13 | 77 | 1.80 |
| Schu $2.4 \times 10^8$ | 4 million | Normal | 10/10 | 100 | 1.70 |
| | | 37 days | 13/14 | 93 | 2.38 |
| | | 34 days | 9/9 | 100 | 2.66 |

* The mice tested were pools of mice which had been immunized with various low-virulent strains and later challenged with the Schu strain. The days indicate the time elapsed since the Schu challenge. The mice were challenged a second time with the 6 strains as indicated.

Serum therapy in infection with the Schu strain.

An attempt was made to alter the course of infection resulting from the injection of the Schu strain mixed with immune serum, by daily injections of additional immune serum. Four groups of 10 mice were inoculated with equal parts of immune serum and decimal saline dilutions of a suspension of Schu cells containing 3.8 - 3800 cells per inoculum. Four groups of 5 mice were inoculated with equal parts of normal serum and cell suspensions corresponding to the above. Daily injections of 0.5 ml of immune serum per mouse were administered to the infected mice. Daily inoculations of normal mouse serum were given to the control mice. The data are presented in Table 10. It is evident from these figures that the survival of mice treated with immune serum was prolonged. The course of infection was not altered although death was delayed.

Table 10

Serum Therapy in Infection with the Schu Strain

| Inoculum with Serum | Test Mice* Dead Tested | Per cent Mortality | Av. Day of Death | MFI | Control Mice** Dead Tested | Per cent Mortality | Av. Day of Death | MFI | PR |
|---|---|---|---|---|---|---|---|---|---|
| 3800 Schu | 10/10 | 100 | 4.0 | 25.0 | 5/5 | 100 | 3.2 | 31.2 | 1.24 |
| 380 Schu | 9/9 | 100 | 5.3 | 18.8 | 5/5 | 100 | 3.4 | 29.4 | 1.56 |
| 38 Schu | 10/10 | 100 | 6.1 | 16.3 | 5/5 | 100 | 4.0 | 25.0 | 1.53 |
| 3.8 Schu | 10/10 | 100 | 6.9 | 21.7 | 5/5 | 100 | 4.6 | 21.7 | 1.50 |

* Daily injections of Jap immune serum were made in the test mice which had received an initial injection of immune serum + Schu cells as indicated.

** Daily injections of normal mouse serum were made in the control mice which had received normal serum + Schu cells as indicated.

Comparison of toxic properties of some strains of Bact. tularense.

It was earlier noted in this work that the PR increased as the $LD_{50}$ titer of the strain tested decreased. It was also noted that a direct ratio existed between the $LD_{50}$ and the average day of death. That is, as the $LD_{50}$ titers of the various strains decreased, the average day of death decreased in all but a few of the strains tested. This was calculated on the basis of a comparable number of $LD_{50}$. An attempt was made to determine whether the shorter survival rate was due to the increased number of organisms necessary for a lethal dose or to a toxic property possessed by some strains of Bact. tularense. If the latter supposition were valid, the increase in PR would be due to an anti-toxic capacity of immune serum.

Three groups of 100 mice were injected with sub-lethal doses of 3 strains of Bact. tularense as follows: the first group of 100 mice received $2.0 \times 10^8$ organisms of the HD strain; the second group of 100 mice received $3.6 \times 10^2$ organisms of the RI strain; the third group of 100 mice received $4.8 \times 10^1$ organisms of the Bish strain. The doses employed were slightly larger than a sub-lethal dose in each case. The percentages of mortality in the 3 groups were 44, 17 and 77 per cent, respectively. Two weeks after this immunizing dose each group was subdivided and challenged with

the HD, RI, Bish, and Schu strains. The challenge doses of the 4 strains employed were $3.0 \times 10^9$ HD cells, $1.2 \times 10^7$ RI cells, $4.1 \times 10^6$ Bish cells, and $1.2 \times 10^3$ Schu cells. The first challenge dose comprised approximately 30 $LD_{50}$ doses while the last 3 challenge doses contained approximately 1000 $LD_{50}$ doses. It may be seen from the data presented in Table 11 that the majority of immune mice challenged by the HD strain succumbed in less than 2 days. Very few of the immune mice which were challenged by the RI, Bish, or Schu strains died and the day of death of the fatalities was well over 2 days. The mice which had been immunized by the introduction of viable HD cells withstood challenge with the Schu and Bish strains completely, and showed 93 per cent survival with challenge by the RI strain. This indicated good immunogenic activity in the HD cells. However, it will be noted that 57 per cent of the HD immune mice succumbed to challenge with the homologous strain. This mortality percentage was somewhat lower than the figures obtained against challenge with the RI strain (85 per cent) and with the Bish strain (80 per cent). It is apparent that the HD strain is a good immunizing strain but is able to produce a toxic death in mice immunized by homologous or heterologous strains. It may also be noted that the Bish, Schu and RI strains appear to be very similar in their lack of toxicity and resistance to protection. They differ in this respect from the HD strain.

Table 11

Comparison of Toxicity of Strains of Bact. tularense

| Challenge Strain* and Dose | Immunizing Strain** | Dead Tested | Av. Day of Death | Per cent Mortality |
|---|---|---|---|---|
| $3.0 \times 10^9$ HD | HD | 8/14 | 1.8 | 57 |
| | RI | 17/20 | 1.2 | 85 |
| | Bish | 4/5 | 1.0 | 80 |
| | Normals | 5/5 | 2.0 | 100 |
| $1.2 \times 10^7$ RI | HD | 1/14 | 1.0 | 7 |
| | RI | 1/20 | 4.0 | 5 |
| | Bish | 0/5 | none | 0 |
| | Normals | 5/5 | 3.8 | 100 |
| $4.1 \times 10^6$ Bish | HD | 0/14 | none | 0 |
| | RI | 0/20 | none | 0 |
| | Bish | 1/5 | 2.0 | 20 |
| | Normals | 4/5 | 3.8 | 80 |
| $1.2 \times 10^3$ Schu | HD | 0/13 | none | 0 |
| | RI | 1/23 | 7.0 | 4 |
| | Bish | 2/6 | 5.0 | 33 |
| | Normals | 5/5 | 4.0 | 100 |

* The challenge dose of the strains represented approximately the following $LD_{50}$:

HD-30; RI-1000; Bish-1000; Schu-1000

** The immunizing dose of the strains employed were:

HD-$2 \times 10^8$; RI-$3.6 \times 10^2$; Bish-$4.8 \times 10^1$

Adsorption of the protective substance.

The adsorption process was carried out as previously described. To test the protective capacity of the adsorbed and unadsorbed immune sera, equal volumes of each were added to a $10^{-1}$ dilution of a standard saline suspension of Jap cells and used as inocula for two groups of normal mice. Adsorbed and unadsorbed normal mouse sera were used as controls. The organisms used for adsorption were living Jap cells, boiled Jap cells, or living 38 cells. It is evident from the results shown in Tables 12, 13, and 14, that the protective substance was adsorbed from the immune serum, either by viable or dead cells of the homologous strain, or viable heterologous cells.

Cross-species protection.

It was of interest to note whether immune serum from one species was able to protect another species against lethal infection with Bact. tularense. Immune sera were obtained from the rat, rabbit, guinea pig and man and employed in the routine protection test with the mouse. A brief history of the sera tested is recorded in Table 15. Table 16 presents the results obtained from the sera tested. The PR obtained indicate excellent protective capacity for the sera of the species tested, against infection in the mouse with Bact. tularense.

Killed antigen for the production of protective serum.

From previous work performed in this laboratory (3) it was found that an original inoculum of $4 \times 10^4$ Jap cells multiplies in the mouse to approximately $6.9 \times 10^6$ within a 5 day period. Therefore, an inoculum consisting of this number of boiled Jap cells was used to approximate the height attained by multiplication. In contrast to this suspension of killed cells, a suspension of living Jap cells approximating $4 \times 10^4$ cells per ml was employed for control purposes. A group of 25 mice was inoculated with the boiled Jap cells. Each mouse in this group received $6.3 \times 10^6$ killed Jap cells. A group of 25 mice was inoculated with the suspension of living Jap cells. Each mouse in this group received $3.1 \times 10^4$ living organisms.

On the fifth post-inoculation day 5 mice from each group were challenged for immunity with $2.8 \times 10^3$ cells of the Schu strain. This challenge comprised approximately 2000 $LD_{50}$ doses of the highly virulent strain. There were no survivors in the group of 5 mice which had received the boiled Jap cells. There were no deaths in the group of 5 mice which had received the living Jap cells.

The remaining 20 mice in each group were bled from the heart on the fifth post-inoculation day. The sera which were collected into two pools were added to an equal volume of a $10^{-1}$ dilution of Jap cells for the usual protection

test. The results, which are presented in Table 17, indicate that a protective serum may be produced equally well by either living or killed Jap cells as antigen.

Table 12

Absorption of Protective Substance with Living Jap Cells

| Serum and Challenge Dose | Test Mice: Dead Tested | Test Mice: Per cent Mortality | Test Mice: Av. Day of Death | Test Mice: MPI | Control Mice: Dead Tested | Control Mice: Per cent Mortality | Control Mice: Av. Day of Death | Control Mice: MPI | PE |
|---|---|---|---|---|---|---|---|---|---|
| Immune serum & $2.7 \times 10^8$ Jap | 13/24 | 54 | 5.9 | 9.1 | 4/4 | 100 | 1.0 | 100.0 | 10.98 |
| Absorbed Immune serum & $2.7 \times 10^8$ Jap | 24/24 | 100 | 1.6 | 62.5 | 4/4 | 100 | 1.0 | 100.0 | 1.60 |

Table 13

Absorption of Protective Substance with Boiled Jap Cells

| | Test Mice | | | | Control Mice | | | | PR |
|---|---|---|---|---|---|---|---|---|---|
| Serum and Challenge Dose | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| Immune Serum & $2.8 \times 10^8$ Jap | 15/18 | 83 | 4.4 | 18.8 | 4/4 | 100.0 | 2.0 | 50.0 | 2.65 |
| Absorbed Immune Serum & $2.8 \times 10^8$ Jap | 17/18 | 93 | 1.2 | 77.5 | 4/4 | 100.0 | 1.5 | 66.6 | 0.85 |

Table 14

Adsorption of Protective Substance with Living 38 Cells

| Serum and Challenge Dose | Test Mice: Dead Tested | Test Mice: Per cent Mortality | Test Mice: Av. Day of Death | Test Mice: MPI | Control Mice: Dead Tested | Control Mice: Per cnet Mortality | Control Mice: Av. Day of Death | Control Mice: MPI | PE |
|---|---|---|---|---|---|---|---|---|---|
| Immune Serum & $1 \times 10^3$ Jap | 5/12 | 41 | 6.4 | 6.4 | 4/4 | 100 | 1.8 | 55.5 | 8.67 |
| Absorbed Immune Serum & $1 \times 10^8$ Jap | 11/11 | 100 | 1.4 | 71.4 | 4/4 | 100 | 1.2 | 83.3 | 1.02 |

Table 15

Data Pertaining to Sera Tested

| Type of Serum | Agglutinin Titer | Post-immunization Date of Collection | Storage before Use |
|---|---|---|---|
| Rat | 1:80 | 6 weeks | No storage |
| Rabbit | 1:2560 | ? | 14 months |
| Guinea pig | 1:2560 | 8 weeks | No storage |
| Human | 1:2560 | 4 months after probable sub-clinical infection | No storage |

Table 16

House Protective Capacity of Serum from Various Species

| Serum & Dose | Test Mice | | | | Control Mice | | | | PR |
|---|---|---|---|---|---|---|---|---|---|
| | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| Rat ≠ 2.7 x $10^9$ Jap Cells | 17/20 | 85 | 5.0 | 17.0 | 10/10 | 100 | 1.2 | 83.3 | 4.90 |
| Rat ≠ 2.7 x $10^8$ Jap Cells | 8/20 | 40 | 5.6 | 7.2 | 10/10 | 100 | 1.6 | 62.5 | 8.68 |
| Rabbit ≠ 3 x $10^8$ Jap Cells | 18/20 | 90 | 4.2 | 21.4 | 10/10 | 100 | 1.3 | 76.9 | 3.59 |
| Rabbit ≠ 3 x $10^7$ Jap Cells | 7/20 | 35 | 6.7 | 5.2 | 9/10 | 90 | 3.0 | 30.0 | 5.74 |
| Guinea Pig ≠ 4.4 x $10^9$ Jap Cells | 13/20 | 65 | 5.3 | 12.2 | 5/5 | 100 | 1.0 | 100.0 | 8.11 |
| Guinea Pig ≠ 4.4 x $10^8$ Jap Cells | 6/19 | 31 | 2.3 | 13.5 | 10/10 | 100 | 1.3 | 76.9 | 5.69 |
| Human ≠ 1.5 x $10^8$ Jap Cells | 11/20 | 55 | 5.3 | 10.3 | 6/6 | 100 | 2.0 | 50.0 | 4.85 |

Table 17

Production of the Protective Substance with Boiled Jap Antigen

| Immune Serum | Test Mice | | | | Control Mice | | | | PR |
|---|---|---|---|---|---|---|---|---|---|
| | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| From Boiled Jap Cells* + $1 \times 10^8$ Jap | 17/20 | 85 | 4.0 | 21.2 | 4/4 | 100 | 1.8 | 55.5 | 2.61 |
| From Living Jap Cells** + $1 \times 10^8$ Jap | 15/20 | 75 | 5.2 | 14.4 | 4/4 | 100 | 1.8 | 55.5 | 3.85 |

* The immune serum was obtained from mice inoculated with Jap cells which had been boiled for 10 minutes ($3.1 \times 10^6$).

** The immune serum was obtained from mice inoculated with living untreated Jap cells ($3.1 \times 10^4$).

Avirulent strain 38 as antigen for the production of the protective substance.

It has long been known that the 38 strain of Bact. tularense is incapable of producing a clinical infection in the white mouse. It has recently been shown, however, that this avirulent strain is capable of a fair degree of multiplication in the white mouse (3). It was of interest, therefore, to use this strain in the protection test and to compare its activity in this light with a strain of known infectivity. Accordingly, two groups of mice were inoculated with 1 x $10^4$ Jap cells and 1 x $10^8$ 38 cells. Both groups of mice were bled from the heart on the fifth post-inoculation day and the routine protection test was carried out on the two pools of immune sera. The results obtained, as indicated in Table 18, showed good protection from both pools of sera. The two-fold increase in protection demonstrated by serum obtained from mice inoculated with 38 cells over the Jap immune serum may be of some significance because of the magnitude of the protection afforded. The degree of protection is variable, however, and open to question. The results indicate that both sera were highly protective. A very low mortality occurred in both groups of mice. The average days of death of the fatalities were greatly prolonged.

Table 18

Comparison of Jap and 38 Strains of Bact. tularense as

Antigen in the Mouse Protection Test

| | Test Mice | | | | Control Mice | | | | PR |
|---|---|---|---|---|---|---|---|---|---|
| Serum and Jap Cells | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| Jap Serum* ≠ $1 \times 10^8$ | 7/24 | 29 | 5.4 | 5.0 | 6/6 | 100 | 1.3 | 76.9 | 15.38 |
| 38 Serum** ≠ $1 \times 10^8$ | 2/17 | 11 | 4.5 | 2.4 | | | | | 32.04 |

* The Jap serum was obtained from mice inoculated with the Jap strain.

** The 38 serum was obtained from mice inoculated with the 38 strain.

Sensitized antigen for the production of the protective substance.

One ml of a saline suspension of a 24 hour culture of Jap cells approximating 2 times a standard suspension was added to 4 ml of immune serum collected on the fifth post-immunization day. This serum-cell suspension was refrigerated at 4° C overnight. The following day the cells were reclaimed by centrifugation and made up in saline to give 24 per cent light transmission on the Coleman spectrophotometer at a wave length of 600. Decimal saline dilutions of the standard suspension were prepared and plate counts made on the $10^{-6}$ and $10^{-7}$ dilution according to the method of Downs et al (12). The same procedure was carried out on a suspension of cells using normal serum in place of the immune serum.

Two groups of 34 mice were inoculated, intraperitoneally, with the cells sensitized with immune serum and with the cells treated with normal serum, respectively. The inocula consisted of $4 \times 10^3$ sensitized cells and $2 \times 10^4$ unsensitized cells. On the fifth post-immunization day, 7 mice in each group were challenged with $2 \times 10^3$ cells of the virulent Schu strain. This challenge consisted of approximately 2000 $LD_{50}$ doses. In the group of mice which received the sensitized cells, 2 out of 7 mice died, giving a 71 per cent survival rate. In the group of mice which received the cells treated with normal serum, 1 out of 7 mice died, giving

a survival of 85 per cent. The difference in survival rates of the two groups is insignificant and shows good immunity in both groups.

On the fourth post-immunization day, 2 mice from each group were sacrificed and quantitative spleen counts were made. The counts indicated invasion of Bact. tularense into the spleen of each mouse tested. This signified that multiplication of the organism was not inhibited by treatment of the cells with immune or normal serum.

The remaining 25 mice from each group were bled from the heart and the serum pooled into 2 groups. An equal amount of a $10^{-1}$ dilution of Jap cells was added to the serum obtained from mice receiving sensitized cells and to the serum obtained from mice receiving cells treated with normal serum. The 2 serum-cell suspensions were inoculated into 2 groups of normal mice. The results, presented in Table 19, showed equal protection derived from serum produced by sensitized cells as antigen or unsensitized cells as antigen.

Minimal amount of antigen necessary to produce the protective substance.

In order to determine the least amount of antigen necessary to produce a protective serum in the white mouse, serial 10 fold saline dilutions of a standard suspension of Jap cells were employed as inocula. The dilutions used ranged from a $10^{-5}$ dilution to a $10^{-9}$ dilution. Five days

following the intraperitoneal injection of these amounts of cells into 5 groups of mice, the sera were collected and used in the routine mouse protection test. The data from Table 20 indicate that a definite threshold exists as a minimal inoculum essential for the production of a protective serum. A PR of 5.6 was obtained from serum produced by the injection of $6.7 \times 10^2$ cells. When $6.7 \times 10^1$ cells were used as the immunizing dose, the PR dropped dramatically to 0.69. A PR value of less than unity indicates that the experimental mice died more rapidly than the control mice and thus were not protected in any degree. The data also indicate some relationship between increasing amounts of antigen and increasing PR.

Table 19

Sensitized Antigen for the Production of the Protective Substance

| Test Mice | | | | | Control Mice | | | | PR |
|---|---|---|---|---|---|---|---|---|---|
| Immune Serum | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| From sensitized cells + $4.8 \times 10^8$ Jap* | 16/25 | 64 | 4.6 | 13.9 | 6/6 | 100 | 2.0 | 50.0 | 3.59 |
| From unsensitized cells + $4.8 \times 10^8$ Jap** | 15/22 | 68 | 6.0 | 11.3 | 6/6 | 100 | 2.0 | 50.0 | 4.42 |

* The immune serum was obtained from mice inoculated with Jap cells which had been treated with immune Jap serum before injection.

** The immune serum was obtained from mice inoculated with Jap cells which had been treated with normal serum before injection.

Table 20

Minimal Effective Inoculum of Antigen for the Production of the Protective Substance

| Amount of Antigen | Immune Serum with Challenge Dose | Head Tested | Per cent Mortality | Av. Day of Death | MPI | PR |
|---|---|---|---|---|---|---|
| $6.7 \times 10^4$ Jap | 0.5 ml + 0.5 ml $1 \times 10^8$ Jap | 3/7 | 43 | 5.3 | 8.1 | 6.62 |
| $6.7 \times 10^3$ Jap | " | 2/4 | 50 | 5.5 | 9.0 | 5.96 |
| $6.7 \times 10^2$ Jap | " | 4/7 | 58 | 6.0 | 9.6 | 5.59 |
| $6.7 \times 10^1$ Jap | " | 9/9 | 100 | 1.3 | 76.9 | 0.69 |
| 6.7 Jap | " | 9/9 | 100 | 1.4 | 71.4 | 0.75 |
| - - - - | 0.5 ml normal serum + 0.5 ml $1 \times 10^8$ Jap | 6/7 | 86 | 1.6 | 53.7 | - - - |

Effect of alteration in the routes of inoculation on protection.

The usual manner of inoculation of serum and challenge dose in the mouse protection tests was the intraperitoneal inoculation of equal parts of the mixture. In order to establish the fact that protection from immune serum was not due to sensitization of cells for phagocytosis, the following inoculations were made. To the first group of normal mice, a mixture of 0.5 ml immune serum and 0.5 ml 1 x $10^8$ Jap cells was given intraperitoneally to each mouse. In the second group of normal mice, each mouse received 0.5 ml immune serum intraperitoneally into the lower left quadrant followed immediately by the injection of 0.5 ml 1 x $10^8$ Jap cells subcutaneously over the lower right quadrant. Each mouse in the third group was given 0.5 ml immune serum subcutaneously over the lower left quadrant followed immediately by the subcutaneous injection of 0.5 ml 1 x $10^8$ Jap cells over the lower right quadrant. The groups of control mice received normal mouse serum and organisms by identical routes of inoculation. The results are presented in Table 21. It is seen that the protection derived from immune serum is unaltered by the route of inoculation employed. It seems unlikely, therefore, that protection is due solely to the clumping of sensitized cells for the enhancement of phagocytosis.

Table 21

Effect of Alteration in Routes of Inoculation on Protection

| | Test Mice* | | | | Control Mice** | | | | PR |
|---|---|---|---|---|---|---|---|---|---|
| Route | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | |
| Serum ip Cells ip opposite sides | 7/20 | 35 | 5.0 | 7.0 | 6/6 | 100 | 1.5 | 66.6 | 9.51 |
| Serum ip Cells sq | 7/20 | 35 | 4.4 | 7.9 | 6/6 | 100 | 1.6 | 62.5 | 7.91 |
| Serum sq Cells sq | 10/20 | 50 | 5.7 | 8.7 | 6/6 | 100 | 1.6 | 62.5 | 7.16 |
| Serum ip Cells ip together | 12/18 | 66 | 5.3 | 12.5 | 6/6 | 100 | 1.5 | 66.6 | 5.32 |

* Test mice received immune serum + 1 x $10^8$ Jap cells by the route indicated.

** Control mice received normal serum + 1 x $10^8$ Jap cells by the route indicated.

Comparison of protection given by spleen extracts and serum obtained from mice receiving viable or phenolized cells.

A group of mice was inoculated with a $1 \times 10^{-5}$ dilution of a standard suspension of viable Jap cells. Six days after this inoculation the group of mice was bled and the serum was pooled for the usual mouse protection test. Immediately after death the spleens from these mice were collected and frozen. An extract of the spleens was made as described above. A second group of mice was inoculated with a $1 \times 10^{-2}$ dilution of a standard suspension of phenolized Jap cells. Six days after this inoculation this group of mice was bled and the serum was used for a mouse protection test. Immediately after death the spleens of these mice were collected, frozen, and later extracted with saline as previously described. Control mice received normal serum and an extract of normal mouse spleens with the challenge dose. The spleen extracts were diluted to give 0.25 g per ml of saline. Each mouse received 0.5 ml of the extract which corresponded to 0.125 g of spleen plus 0.5 ml of a $1 \times 10^{8}$ Jap cell suspension. The results presented in Table 22 indicate that no protection was provided by the spleen extracts. There was no significant difference in the protection derived from serum obtained from mice inoculated with viable cells or with phenolized cells.

Table 22

Comparison of Protection Given by Spleen Extracts and Serum from Mice Receiving Viable or Phenolized Cells

| Inoculum ≠ Jap Cells | Test Mice: Dead Tested | Test Mice: Per cent Mortality | Test Mice: Av. Day of Death | Test Mice: MPI | Control Mice: Dead Tested | Control Mice: Per cent Mortality | Control Mice: Av. Day of Death | Control Mice: MPI | PR |
|---|---|---|---|---|---|---|---|---|---|
| Group I | | | | | | | | | |
| Serum ≠ $1 \times 10^8$ | 9/20 | 45 | 5.7 | 7.8 | 11/11 | 100 | 3.1 | 32.2 | 4.12 |
| Extract ≠ $1 \times 10^8$ | 18/20 | 81 | 4.0 | 20.5 | 10/10 | 100 | 3.2 | 31.2 | 1.52 |
| Group II | | | | | | | | | |
| Serum ≠ $1 \times 10^8$ | 6/19 | 31 | 6.1 | 5.0 | | | | | 6.44 |
| Extract ≠ $1 \times 10^8$ | 10/12 | 83 | 3.3 | 25.1 | | | | | 1.24 |

Group I – The serum and spleen extract used in this group were obtained from mice receiving viable Jap cells.

Group II – The serum and spleen extract used in this group were obtained from mice receiving phenolized Jap cells.

## Discussion

It is obvious from the results obtained throughout this study that although immune mouse serum is protective, a variation appears in the degree of protection afforded by immune serum. This variation probably is due to the protective antibody content of the immune serum. The white mouse appears to vary in its response to the protective antigen as greatly as it does to an agglutinogen. When serum, which was collected from large groups of immune mice, was used as the source of protective antibody for large groups of normal mice, a more reliable trend was obtained. An arbitrary limit was set for an acceptable numerical expression of protection. It appeared from the many results obtained that a PR of 2 was the least sign of protection that could be recognized as significant. A rough statistical analysis of 46 experiments gave an arithmetic mean of the PR as 6.5±0.78. The average MPI obtained for the infected mice was 15.2 and for the control mice, 57.6. More significance was attached to the ratio (PR) obtained by a comparison of the indices of control and infected mice than the MPI of either alone. Meyer (13) has stated that from experiments on animals with the plague bacillus the protective index should be at least one-half of the normal index in order to obtain a high rate of survival from infections which are invariably fatal to controls. He also found (11) that an MPI below 10 in guinea

pigs indicates a high percentage of survival in massive infections with the plague bacillus. In the case of Bact. tularense a lethal challenge dose of the Jap strain usually killed 100 per cent of the controls in 1 to 2 days. Immune serum was rarely able to protect 100 per cent of the infected mice against this dose but the survival time and the survival percentage were increased noticeably from that of the control animals. In the immunological studies no attempt was made to correlate the degree of protection with the potency of the serum. The test was used as a measure in determining the presence or absence of protection.

It was of interest to apply the calculations and interpretations used in this work to that of others. In a recent report (14) on the protection of rats with human and hyper-immune goat serum, figures were presented which showed the day of death of the rats, the total number of deaths, and the percentage mortality. In this test, vaccinated pooled serum, hyper-immune goat serum, and adsorbed hyper-immune goat serum were tested against controls consisting of normal human pooled serum and no serum. The conclusions derived from these results were that the hyper-immune goat serum showed more protection than the vaccinated pooled serum and the adsorbed hyper-immune goat serum had been relieved of its protective capacity by the adsorption process with a phenol extracted polysaccharide of Bact. tularense.

Since two controls were used in this work, there are two PR values calculated in the manner followed in the mouse protection test carried on in this laboratory. The applications are presented in Table 23. It is evident from an observation of this table that the mathematical manipulations clearly emphasize the conclusions which that author presented. Of the two PR calculated from her results, the ratio determined by comparison of the infected rats receiving immune serum and those receiving none was greater than that compared to the rats receiving normal serum. It appears that normal serum has some influence on the pathogenesis of the organisms. Therefore, it is logical that the PR computed on this basis is more acceptable of the true result than a PR computed without taking this factor into consideration. The PR obtained are comparable to those computed in this study and are indicative of good protection.

It was of interest to compare the appearance, duration and response to a second inoculation, of the protective antibody and the agglutinating antibody. It was found (Table 1 and Figure 1) that the protective antibody appeared in the circulating blood between the third and fourth day. Demonstrable agglutinins did not appear until the fifth or sixth day following injection. These results were obtained in whole blood and in serum. Whole blood gave no better protection than immune serum. It was also found that the protective capacity of immune serum lasted at least

Table 23

Calculations of Protection Tests Applied to Results Obtained from an Outside Source.[1]

| Serum | Dead Tested | Per cent Mortality | Av. Day of Death | MPI | PR* | PR** |
|---|---|---|---|---|---|---|
| Normal human pool | 14/20 | 70 | 7.1 | 9.8 | — | — |
| Vaccinated pool | 11/20 | 55 | 10.5 | 5.2 | 1.8 | 3.4 |
| Hyper-immune goat serum, 1:5 dil. | 4/20 | 20 | 11.5 | 1.7 | 4.5 | 10.5 |
| Absorbed goat serum, 1:5 dil. | 14/20 | 70 | 6.5 | 10.7 | .9 | 1.6 |
| None | 19/20 | 95 | 5.3 | 17.9 | — | — |

1 Results taken from "The Immune Response of Man to Pasteurella tularensis." Mary Mitchell Alexander. Ph.D. Thesis, The George Washington University, 1949.

* PR = MPI of normal serum / MPI of immune serum

** PR = MPI of no serum / MPI of immune serum

7 weeks as contrasted to 15 days for demonstrable agglutinins following one injection of 4 x $10^4$ Jap cells (Table 2 and Figure 2). The agglutinin response was immediate following a second inoculation but a rise in the protective antibody content was not apparent for another 3 days. As seen in Table 4 and Figure 4, the response of agglutinating antibodies, in general, was not as great following the "booster dose" as the initial response. These results indicate that the 2 antibodies are a separate response, and that the protection derived from immune serum is not correlated with the agglutination titer. These findings are in agreement with the results obtained by other workers (1, 8,9) who have found that there is little correlation between the agglutinating capacity and the protective capacity of immune serum. Ruchman and Foshay (1) have reported on an intensive study of the immune response in mice after vaccination with Bact. tularense. Following a single dose of a phenolized suspension of the Schu strain they found that white mice developed a maximal agglutinative titer of 1:40 to 1:80 at 3 to 4 weeks. A slight negative phase followed by a peak of 1:640 to 1:1280 in 1 week developed from a "booster dose" administered 6 to 7 weeks after the initial vaccination. No resistance could be demonstrated in these mice against small numbers of living, virulent cells in spite of the accelerated immune response. The

titers reported by these workers were considerably higher than those obtained in this laboratory resulting from the injection of a sub-lethal dose of living cells. The immune response in the mice observed in this study appeared much earlier than the time reported by these workers and disappeared earlier. The passive immunization used in this study showed no correlation with the agglutinin content of the immune serum and this conclusion is in agreement with results cited. It has been shown that the immune state in the white mouse becomes evident 3 days after the immunizing injection (3). This time coincides with the appearance of protective antibodies and suggests a close relation between active and passive immunity. It seems evident that the protective antibody is one part of the immunity mechanism in the white mouse. The protective antibody content of immune serum apparently is not related to the agglutinin content.

An attempt was made to determine the time of appearance of immunity in the passively protected mouse. Downs and Woodward had previously determined that immunity appears 3 days after an inoculation of a sub-lethal dose of viable Jap cells (3). Their results were determined by daily inoculation of 100 $LD_{50}$ of the Schu strain. In this study, daily challenge doses of 1000 $LD_{50}$ of the Schu strain were made in mice which had received 100 $LD_{50}$ of the Jap strain together with immune serum. It was

shown (3) that multiplication of the Jap cell in the white mouse reached a peak on the second to fifth day and that the peak of multiplication was reached more quickly and was higher when larger numbers of organisms were injected. The extent of multiplication of the Jap cell in the host when administered in the presence of immune serum is unknown. It is known, however, that the rate of multiplication of the Jap cell in the actively immunized mouse is slower than in the normal mouse and the peak of multiplication is lower. It can be assumed that multiplication of the organism takes place in the presence of immune serum from the fact that at least 50 per cent of the protected mice die from infection. In this study, an over-whelming dose of Schu cells was injected into a mouse which was already inoculated with a lethal dose of Jap cells 1 to 4 days previously. It is obvious that the superimposed infection injected during the 2 to 4 day period of rapid multiplication is cumulative in its effect. An analysis of Table 5 shows 80 to 90 per cent of the test mice died in approximately the same length of time as the control mice on the second, third, and fourth days. It is believed that these results are not a true picture from which the development of immunity can be determined. The results are probably confused by the massive challenge dose employed. However, it is evident that by the fifth day the immunity mechanisms are organized and sufficiently effective to delay the average day of death

3 days longer than that of the control mice. The immunity mechanisms were not sufficiently effective, however, to save more than 20 per cent of the test mice. It is known from this experiment and from other experiments, that a solid immunity may be demonstrated against a challenge dose of 1000 $LD_{50}$ Schu cells in mice which have survived 10 days. It is probable that the development of immunity commences before the fifth day in the passively protected mouse and coincides with the development of immunity in the mouse receiving sub-lethal immunizing doses.

A striking similarity exists between the minimal effective antigen for the production of active and passive immunity. It was previously determined in this laboratory (3) that 200 Jap cells as the initial dose served to confer a solid immunity to infection. From Table 20 it is seen that 600 Jap cells was the minimal effective inoculum for the production of the protective substance. This number represents the threshold of stimulation below which no protection may be demonstrated. Since the work cited (3) indicated that stimulation of an immune response was possible with 20 Jap cells, it is apparent that the protection test is not sufficiently sensitive to detect the slight degree of immunity elicited by the decreased dose of cells. Since the inoculum essential for the production of a solid active immunity is in the same order of magnitude as that necessary for the production of protective antibodies, it would again

appear that the protective antibodies are an important part of the defense mechanism of the white mouse. It is also evident that a definite amount of antigen is essential for immunogenic purposes. Although no differences in antigenic structure of Bact. tularense have been demonstrated by workers in this field, it is questionable that each strain of Bact. tularense possesses identical quantities of the immunogenic antigen. It is possible, therefore, that the strains which have been reported as poor immunizing agents (3) are deficient in quantity of the immunogenic antigen. This has been proven to be the case with the avirulent strains of the plague bacillus (13). Antigen analysis of the different avirulent strains of poor immunogenicity in mice were shown to vary significantly in the content of fraction IB which is believed to be responsible for this activity.

It was found that 0.01 ml of immune serum was the least amount of serum that could show significant protection. As seen in Table 3, amounts greater than 0.01 ml did not increase the degree of protection significantly. This indicates that a certain quantity of serum is necessary to neutralize the lethal effect of a constant challenge dose.

From the experiments conducted on the protection of Jap immune serum against heterologous strains, it was found that, in a general way, the PR increased as the

virulence, in terms of $LD_{50}$ titers, decreased. At first glance this appeared to indicate that the protection of immune serum was simply insufficient to neutralize the potency of the factors concerned in the highly virulent strains. However, it was soon realized that immune serum could neutralize, or at least show some effect against 1000 $LD_{50}$ of some strains and fail completely to affect 1 $LD_{50}$ of another strain. It was found that good protection was afforded by immune serum against some of the low-virulent strains, especially those strains which have the capacity to overwhelm the host in less than 24 hours. An excellent example of this protection is seen in Tables 7 and 8 where immune serum protects "completely" against a challenge dose with the Schad strain. This strain has an $LD_{50}$ titer of $10^{-0.5}$. An inoculum consisting of 1 $LD_{50}$ will kill approximately half of the control mice. The mice which die are killed in less than 24 hours. Organisms may be recovered from the spleen and heart blood after death, indicating multiplication and invasion. Immune serum is able to protect completely against this sudden overwhelming infection produced by the introduction of approximately 1 billion organisms. In contrast to the effectiveness of immune serum in controlling the course of infection produced by this strain, immune serum is unable to prevent 1 Schu cell from becoming established in the mouse and leading to eventual death.

Two possibilities for the explanation of this contrast were brought forward. The first was a question of a toxic factor present in some strains of Bact. tularense, or possibly, all strains including the Schu strain. The effectiveness of immune serum then, would be due to neutralization of this toxin. Upon the introduction of a very few cells of the Schu strain into the susceptible mouse, a delay of approximately 4 days is noticed until the infected animal shows any symptoms of the disease. If the Schu strain also contains the hypothetical toxin, it is not manifested until the original inoculum has multiplied to a level of approximately $10^7$ cells. At this time, 4-5 days after inoculation, the mouse may then be subjected to a lethal amount of toxin. Theoretically, this lethal amount of toxin is contained in, or excreted by a suspension of cells in the order of $10^8$ cells per ml of inoculum. If this assumption were valid, death of the mouse 5 days following the injection of 1 Schu cell would be comparable to the rapid death observed after the injection of $10^8$ Jap cells or $10^9$ Schad cells. The cause of death in these tularemic infections would then be due to a toxic factor which opens a pathway for an overwhelming septicemia.

The second possibility to explain the lack of protection of immune serum against the Schu strain is the conjecture that the Schu strain contains no toxic-like antigen.

It might differ from the low-virulent strains by the possession of an unknown antigen not capable of producing a neutralising antibody that is transferable in passive protection. The response to this unknown antigen would necessarily be a tissue alteration, evident in the immune mouse, but not transferrable or demonstrable by known serological means. The cause of death from injection of a few Schu cells would be due to the ability of these cells to multiply and invade the inexperienced host uninterruptedly.

In order to substantiate either hypothesis, an attempt was made to control infection from the Schu strain by serum therapy. Daily inoculations of immune serum were administered following the challenge dose. Although death of the infected animals was delayed, no evidence of the usual type of protection was noted. If the Schu strain releases a toxin which accumulates as the invading organisms multiply, it cannot be neutralized by serum therapy. The daily administration of immune serum was in quantity sufficient to protect against an inoculum of $5 \times 10^8$ Jap cells. This inoculum can be controlled by as little as 0.01 ml of immune serum. In this experiment, 3 Schu cells were able to become established in the host in the presence of quantities of immune serum. It seems reasonable to assume that if a toxin were present, an antitoxin did not interfere with multiplication of the Schu cells. A similar condition has been

reported by Meyer et al (15) from their work on the plague bacillus. They found that highly potent antiplague rabbit serum used in the treatment of pneumonic plague, tended to localize the infection into abscess-like areas but failed to retard multiplication of the bacilli, and eventually failed to prevent the damaging effects of the toxins. An exact comparison of the mechanisms involved in two infections such as tularemia and plague may not be justifiable because of the paucity of our present knowledge concerning the antigenic complex of Bact. tularense.

Evidence accumulated in this study points toward the possession of at least two antigens in the strains of Bact. tularense. This evidence is derived mainly from a comparison of the strains possessing a low $LD_{50}$ titer and the ability to kill the experimental host quickly with the contrasting strains. A clearer interpretation of the results is obtained by representing an antigenic complex involving two factors. If the symbol "M" is used to signify a multiplication factor, and "T" a toxin factor, then the low-virulent but quick killing strains, such as the Schad strain, would be designated as "mT." This symbol would imply a major toxin antigen and a minor multiplication antigen. Similarly, the strains of moderate virulence and quick killing time, such as the Jap strain, would be designated as "MT." This strain has both the ability to multiply quickly and to kill

quickly. On the other hand, the Schu strain would be designated as "Mt," because of its rapid multiplication and relatively slow killing time.

The "T" antibody appears to be a stable substance which is not destroyed by heating to 65° C, freezing and thawing, storage at room temperature or at 4° C for several months. It may be produced by the "T" antigen which has been boiled, phenolized or formalinized. It is antigenic in the mouse, rat, rabbit, guinea pig or human. The antibody is demonstrable by passive immunization against the strains which possess the toxic factor. The antibody appears to be one part of the entire defense.

The "M" antigen is necessarily active only in the viable tularense cell. The introduction of this antigen appears to be essential for the production of the immune state in the host. Since it is essential to have multiplication occur within the host in order to produce the refractory state, it is evident that some alteration in the host occurs. This alteration is not transferable, or, at least, can not be demonstrated by passive protection with spleen extracts. It is demonstrable by challenge for active immunity. A strain, such as the Jap strain, which possesses both the "M" and "T" factors should produce in the host, both active immunity and serum capable of passively protecting a normal mouse. Such is the case. After treatment with

heat, phenol, or formalin, the dead Jap organism has lost the "M" antigen and cannot produce active immunity in the host. However, the treated organism still retains the "T" antigen and can produce a protective antibody in the host. This protection is demonstrable against the strains of Bact. tularense which rely primarily upon their toxic properties to make the pathway for multiplication, invasion, and death. The Schu strain appears to rely solely on its multiplication factor and the course of infection is little influenced by an anti-toxic protective serum. As seen in Table 11, a challenge of 1000 $LD_{50}$ of the highly virulent Schu strain did not infect mice immunized with the HD strain. On the contrary, a challenge of 30 $LD_{50}$ of the HD strain killed 57 per cent of the mice immunized with HD, 85 per cent of the mice immunized with RI, and 80 per cent of the mice immunized with the Bish strain. All of these mice succumbed in less than 40 hours. The RI and Bish strains were selected for immunization because it is believed that these strains possess little or none of the toxic factor. The HD strain is believed to possess a great abundance of the "T" factor. It is evident from the data that the HD strain is extremely toxic and capable of killing half of the mice immunized with the homologous strain.

As reported in Tables 17 and 22, the antigen which produces the protective substance is present in living or

dead cells. The injection of heat-killed cells or phenolized cells can produce an antibody which will passively protect a normal mouse against the low- and moderately-virulent strains. However, the mouse which produced this protective antibody is not refractory to a challenge of the Schu strain. It is true that death is prolonged by the slight protection afforded by the one injection of "vaccine." But, by the injection of as little as 200 viable Jap cells as an initial dose the mouse is able to overcome 100 $LD_{50}$ of the Schu strain (3). Without this immunizing dose, the mouse is susceptible to 1 Schu organism. From previous work in this laboratory, it is known that all strains of Bact. tularense multiply in the white mouse (3). The immune state is derived from some process involved during multiplication of the cells within the host. Vaccines prepared from cultures grown in the living cells of embryonated eggs confer no better protection than vaccines prepared from artificial media (16). Returning to the former hypothesis, the "M" antigen produces the immune state in the host by some tissue alteration which is not transferable. The idea of a tissue change is not a new supposition. Dubos (17) has stated, "In addition to the antibodies which have been recognized by in vitro reaction, there may be others which have remained undetected because they can operate only in the internal environment of the host, or because they require the participation of some unknown tissue components.

It is also possible that resistance to infection is in some cases the result of modifications of the tissue cells and that it involves changes in permeability, altered enzyme production, and other modifications of metabolism." The negative results with extracts of spleen obtained from mice immunized with viable cells and with phenolized cells (Table 22) might indicate that protection is not derived from a vital property of the cells transferred, such as an antibody. As Gay and Clarke (18) have shown, it may be due to some stimulation to tissue change such as a cellular mobilization. It is known, in the case of the anthrax bacillus, that the interaction of the growing organisms and the host fluids causes the production of a "protective" antigen (4). This antigen gives rise to an active immunity which cannot be passively transferred. This is cited as another example of tissue alteration produced by infection with a bacterial agent. That a similar change occurs in tissues invaded by multiplying Bact. tularense cannot be stated since it has not yet been demonstrated. The evidence that solid immunity is conferred upon the host after infection, points toward this possibility.

By the use of adsorption tests it was found that viable or killed cells of the homologous or heterologous strain were able to adsorb the protective antibody from immune serum (Tables 12, 13, 14). These results indicate that a common antigen is involved in the production of

protective serum. The fact that the avirulent strain 38 was able to adsorb the protective substance, although this strain cannot produce a clinical infection in the mouse indicates that the antibody is only a part of the complete immunity mechanism in this disease. It has been shown that vaccines prepared from the Sohu strain or the 38 strain are equally effective in the white rat (16). This indicates that 2 such strains which differ widely in virulence, rate of growth, abundance of growth, and, likely, other physiological properties, contain a common antigen. It does not indicate, however, that the vaccines contained the full complement of antigen necessary to produce a solid immunity. It seems, rather, that both vaccines and the protective substance are dealing with only one part of the entire defense mechanism.

The results obtained from the experiments on species specificity showed that the mouse can be equally well protected by immune sera produced in the rat, rabbit, guinea pig, man or mouse (Table 16). The antigen which is responsible for the production of this protective antibody appears, therefore, to show no host immunogenic specificity. Because a significant difference existed in the immunogenic activity of several strains of avirulent plague bacilli for the mouse and for the guinea pig, Meyer, et al (13) were lead to the discovery of the complex antigenic make-up of that

organism. Since no such host immunogenic specificity is apparent for the production of a protective antibody against Bact. tularense, it does not preclude the possibility that antigenic differences between strains exists. It appears, rather, that the antigen responsible for the protective antibody is possessed by several strains in common and is equally active immunogenically in the various species studied.

Exactly what pathway the immunity mechanisms follow in a tularemic infection is not clear. That the immune serum acts solely as an opsonin, is not true in the case of tularemia. The evidence presented in Table 21 shows clearly that the route of inoculation of serum and organisms does not alter the protection afforded by the immune serum. When the immune serum was introduced separately and distant from the site of the inoculation of the organisms, there was little chance for the process of sensitization of the organisms leading to immediate phagocytosis. Any attempts to demonstrate an in vitro bacteriostatic or lytic effect of immune serum on the cells have proved futile so far in this laboratory.[1] As yet, there has been no change observed in the oxygen uptake of respiring Bact. tularense cells in the presence of immune serum by manometric techniques. It is possible that control of toxicity and an unknown tissue

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1 Personal communication.

response are the major defense mechanisms against a tularemic infection.

It seems possible to differentiate between strains of Bact. tularense by their behaviour in the mouse, and from the results of the mouse protection test. It has been shown that immune serum does not protect against challenge doses of the Schu, Bish or RI strains. Immune serum protects well against the Jap and Russ strains and completely against such strains as Schad and HD. The strains Schu, Bish and RI, when compared by lethal doses, kill the infected host relatively slowly. The remaining strains cited produce death rapidly. It was seen in Table 9 that 4 million $LD_{50}$ of the Schu strain killed 10 normal mice in an average of 1.7 days. This dose contained $2.45 \times 10^8$ cells. It was also seen that 10 $LD_{50}$ of the Schad strain containing $54 \times 10^8$ cells killed 7 out of 10 normal mice in an average of 1.0 day. The challenge dose of the Schad strain contained only 20 times as many cells as the Schu challenge but 400,000 less $LD_{50}$. Nevertheless, the Schad strain killed the normal mice more quickly. It is possible that all strains of Bact. tularense possess a toxic factor which becomes evident when administered in massive numbers of cells. The difference in strains then appears to be in the quantity of the toxic factor present in the cell. The same is true of the multiplication factor. Some strains, possessing a greater

abundance of this factor, are able to multiply more rapidly in the susceptible host. The difference in immunogenicity of various strains may be due to the quantity of this factor present in the cell. It seems evident that the various strains of Bact. tularense differ in the quantity of these two factors which govern the behaviour of the strains in the white mouse.

## Conclusions

1. A mouse protection test was presented which is repeatable, reliable, and a useful tool for the study of immunological problems in tularemia.

2. Evidence of a protective substance in immune serum against low- and moderately-virulent strains of Bact. tularense was presented.

3. The protective substance appears in immune serum before agglutinins, persists longer than agglutinins, and appears to be separate from agglutinins.

4. The agglutinins gave an anamnestic response to a "booster dose" of Bact. tularense, but the protective antibody responded slowly.

5. The time of appearance of immunity and protective antibody coincide.

6. The protective antibody is heat stable.

7. Protection derived from immune serum is one part of the complete defense mechanism against tularemic infections.

8. Evidence presented indicates that some strains of Bact. tularense possess a toxic factor as well as a multiplication factor.

9. The protective capacity of immune serum in passive immunization may be due to control of the toxic factor possessed by some strains of Bact. tularense.

10. The protective substance is adsorbed by viable or killed homologous or heterologous strains of Bact. tularense.

11. Passive immunization of the white mouse was accomplishe by sera of other species of animals.

12. The protective antibody was produced by viable or killed organisms, by sensitized organisms, or by the avirulent strain of Bact. tularense.

13. The minimal amount of antigen essential for production of the protective antibody coincides with the minimal immunogenic inoculum.

14. The minimal amount of serum capable of protection against a standard inoculum of organisms was determined.

15. Alteration in the routes of inoculation did not affect protection.

16. Spleen extracts do not confer passive immunity.

17. The role of toxic and multiplication factors in virulence of Bact. tularense was discussed.

## Bibliography


1. Ruchman, I. and Foshay, L., 1949. Immune response in mice after vaccination with Bacterium tularense. J. Immunol. 61, 229-234.

2. Coriell, L. L., Downs, C. M., and Clapp, M. P., 1947. Studies on tularemia. III Immunization of mice. J. Immunol. 56, 245-253.

3. Downs, C. M., and Woodward, J. M., 1949. Studies on pathogenisis and immunity in tularemia. III Immunogenic properties for the white mouse of various strains of Bacterium tularense. J. Immunol. 63, 147-163.

4. Watson, D. W., Cromartie, W. J., Bloom, W. L., Heckly, R. J., McGhee, W. J., and Weissman, N., 1947. Studies on infection with Bacillus anthracis. V The isolation of an inflammatory factor from crude extracts of lesions of Bacillus anthracis infection and its biological and chemical relationship to glutamyl polypeptide. J. Inf. Dis. 80, 121-136.

5. Gladstone, G. P., 1946. Immunity to anthrax: Protective antigen present in cell-free culture filtrates. Brit. J. Exp. Path. 27, 394-418.

6. Watson, D. W., Cromartie, W. J., Bloom, W. L., Kegeles, G., and Heckly, R. J., 1947. Studies on infection with Bacillus anthracis. III Chemical and immunological properties of the protective antigen in crude extracts of skin lesions of Bacillus anthracis. J. Inf. Dis. 80, 28-40.

7. Baker, E. E., Sommer, H., Foster, L. E., Meyer, E., and Meyer, K. F., 1947. Antigenic structure of Pasteurella pestis and the isolation of a crystalline antigen. Proc. Soc. Exp. Biol. Med. 64, 139-141.

8. Foshay, L., Ruchman, I., and Nicholes, P. S., 1947. Antitularense serum: Correlation between protective capacity for white rats and precipitable antibody content. J. Clin. Invest., 26, 756-760.

9. Francis, E., and Felton, L. D., 1942. Antitularemic serum. Pub. Hlth. Repts. 57, 44-55.

10. Larson, C. L., 1947. A serum protection test in tularemic infections in the white rat. Pub. Hlth. Repts. 62, 1793-1800.

11. Meyer, K. F., and Foster, L. E., 1948. Measurement of protective serum antibodies in human volunteers inoculated with plague prophylactics. Stanford Med. Bull. 6, 75-79.

12. Downs, C. M., Coriell, L. L., Chapman, S. S., and Klauber, A., 1947. The cultivation of Bacterium tularense in embryonated eggs. J. Bact. 53, 89-100.

13. Meyer, K. F., Foster, L. E., Baker, E. E., Sommer, H., and Larson, A., 1948. Experimental appraisal of antiplague vaccination with dead virulent and living avirulent plague bacilli. The Proceedings of the Fourth International Congresses on Tropical Medicine and Malaria. Wash. D.C., May 10-18, 1948, pp. 264-274.

14. Alexander, M. M., 1949. The immune response of man to Pasteurella tularensis. Table 7, p. 31. Ph. D. Thesis, The George Washington University.

15. Meyer, K. F., Quan, S. F., and Larson, A., 1948. Prophylactic immunization and specific therapy of experimental pneumonic plague. Am. Rev. Tuberculosis, 57, 312-321.

16. Downs, C. M., Coriell, L. L., Eigelsbach, H. T., Flitt, K. F., Pinchot, G. B., and Owen, B. J., 1947. Studies on tularemia. II Immunization of white rats. J. Immunol. 56, 229-243.

17. Dubos, R. J., 1947. The Bacterial Cell. p. 274. Harvard University Press, Cambridge, Mass.

18. Gay, F. P., and Clarke, A. R., 1930. Enhanced passive immunity to streptococcus infection in rabbits. J. Exp. Med. 52, 95-102.