Immunogenic Properties for the White Mouse of Various Strains of Bacterium tularense

by

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INTRODUCTION

It has been the primary purpose of this investigation to determine whether any antigenic differences exist among various strains of Bacterium tularense and to evaluate the ability of these strains to elicit an immunological response in white mice. Except for a comparison of four strains by reciprocal agglutinin absorption tests performed by Francis and Evans (1) the literature does not report any attempt to differentiate strains of Bacterium tularense by serological or immunological methods.

Foshay (2) and Downs (3) in unpublished experiments could not demonstrate antigenic differences in several strains of this organism using serological methods. Antigenic homogeneity in any group of organisms is the exception rather than the rule and since the strains of Bacterium tularense differ in virulence this raises the question of the relation of virulence to antigenic structure and whether the usual serological tests are adequate to measure antigenic differences in this organism.

The host response to killed Bacterium tularense vaccines varies with the species of animal involved. These vaccines elicit good immunity in humans and in rats, and it is possible to produce high agglutinin titers in rabbits although these animals show little immunity to tularemia following vaccination. Guinea pigs and mice, on the other hand, do not develop significant agglutinin titers, or any appreciable immunity as a result of vaccination. It may be possible to explain this variation by assuming that the methods employed in the preparation of the vaccines may destroy all, or nearly all of the immunizing antigen to which the mice, guinea pigs, and rabbits would otherwise react. Rats and humans may either respond to small amounts of the immunizing fraction or develop

immunity through the influence of the denatured antigen. It is also possible that they are immunized by certain cellular constituents to which the other animals do not respond.

The immunogenic properties of killed Bacterium tularense vaccines have been demonstrated in man by Foshay (4) and Kadull et.al. (5) but these vaccines do not produce as solid immunity as that observed following infection with this organism. Killed vaccines protect rats against as much as 1,000,000 LD_{50} of fully virulent Bacterium tularense, Downs et al (6). Immunogenic differences have not been shown to exist between strains and it must be assumed that most of the strains are sufficiently similar to protect against subsequent infection, Foshay (4).

On the other hand, it has been demonstrated that white mice are highly susceptible to tularemia and are protected only to a slight degree by the injection of killed vaccines made from a virulent strain of Bacterium tularense, Coriell et al (7). Because of this susceptibility the mouse is a delicate test animal for determining the virulence of the strains of Bacterium tularense and should also be a satisfactory animal on which to test the potency of vaccines. It was felt that the study of strains of low virulence would provide an opportunity to observe the immunologic response of white mice to inoculation of sublethal concentrations of living organisms of various strains of Bacterium tularense. By this means it might be possible to detect antigenic and immunogenic differences as indicated by the ability of these strains to immunize the mice.

A study was also made of the behavior of the living organisms of these strains in mice during the course of infection since it was thought that the rate and extent of multiplication might be an important

consideration in determining the degree of immunity produced. The fact that the different cultures vary widely in virulence strongly suggests some fundamental difference in the metabolic activity of each strain in relation to the specific host, in this case the white mouse.

A review of streptomycin studies with Bacterium tularense performed by Chapman et al (8) indicated that an extension of that work was necessary in order to elucidate the host-parasite relationship that exists between mice and highly virulent Bacterium tularense during and following streptomycin therapy. Accordingly, investigation was directed toward the determination of the immunogenic properties of these strains, as well as their infectivity for untreated mice and for mice subjected to streptomycin treatment following infection.

In addition, an attempt was made to prepare potent killed vaccines from both highly virulent cultures and from strains of lowered virulence. The vaccines tested include phenolized, acetone extracted, sonically extracted, streptomycin killed and sodium fluoride killed preparations. The production of immunizing antigen in animal tissues was also investigated.

REVIEW OF LITERATURE

Relatively few antigenic studies of Bacterium tularense are reported in the literature. Francis and Evans (1) have demonstrated the close serological cross relationships existing between this organism and those of the genus Brucella, and as previously mentioned, have reported that no differences were observed among four strains of Bacterium tularense as demonstrated by reciprocal agglutinin absorption technics. Bond and Downs (9) on the other hand showed that no serological relationship existed between Bacterium tularense and the Pasteurella group, although this organism has been classified as a member of the genus Pasteurella on the basis of the clinical symptoms and histophathological lesions it evokes in infected rodents.

Differentiation of Bacterium tularense by means of cultural characteristics has been shown to be impracticable by Francis (10) and Downs and Bond (11). They report that nearly all strains ferment most of the usual sugars. Other cultural characteristics such as H_2S production in media containing peptone and blood or serum, or in media containing cystine do not serve as a satisfactory basis for classification, Downs and Bond (11).

Other differences such as that of lowered virulence have been observed in freshly isolated strains of Bacterium tularense. Green (12) has isolated strains of low virulence from ruffed grouse. These cultures produced infections in guinea pigs which caused death after an average duration of 15 days, in contrast to the usual average of 6 days when the guinea pigs were infected with highly virulent strains isolated from rabbits. Also, Davis et al (13) have found strains of Bacterium tularense of low virulence in the Rocky Mountain wood tick (Dermacentor andersoni).

The cultures were observed to have a lowered virulence for rabbits and guinea pigs.

Experimentally induced avirulence has been reported by Foshay (14) who refrigerated for ten weeks subcultures of two virulent strains grown on coagulated egg yolk medium. He was then able to demonstrate that these cultures were almost completely avirulent for guinea pigs and white mice.

Much of the above work has laid a foundation for the investigation of the host-parasite relationships that exist between Bacterium tularense and the class Mammalia. The incidence of tularemia among humans is relatively high in certain areas of the United States, and it is even higher in the rabbit and hare populations and in many other rodents, Jellison and Parker (15). Consequently it has been of interest to determine the relationships existing between Bacterium tularense and various laboratory animals.

Lillie and Francis (16) found that white rats were less susceptible to tularemia than were rabbits. In addition, Downs (17) and Lillie and Francis (16) showed that rabbits could not be immunized with killed or attenuated vaccines of Bacterium tularense. More recently Downs et al (18) have made a study of the comparative susceptibility of laboratory animals. They observed a wide range of susceptibility to tularemia in the species tested. Rabbits, mice, guinea pigs, hamsters and cotton rats rarely recovered from an infective dose. The number of virulent organisms constituting an infective dose varied according to species. The mouse apparently succumbed to infection with one organism. The white rat was found to be susceptible to infection but recovered more often than did the previously mentioned animals. Monkeys were easily

infected but showed a high percentage of recovery. Chicks and dogs appeared to be the least susceptible of the animals that were tested.

Almost simultaneously Larson (19) and Downs et al (6) determined that white rats were easily immunized with killed culture vaccines of highly virulent Bacterium tularense, and further observed that the resistance of normal rats to infection with this organism closely paralleled that of humans. These findings indicated that the white rat might be quite satisfactory for conducting extensive investigations of the host-parasite relationship since it was now possible to follow the course of infection in large numbers of normal and immune animals. In direct contrast to these findings Coriell et al (7) showed that only a slight degree of immunity was elicited in white mice immunized with killed vaccines of highly virulent Bacterium tularense. It is of considerable interest to note that Jawetz and Meyer (20) could immunize mice against plague with killed vaccines of Pasteurella pestis while guinea pigs were not immunized by this method. On the other hand, a small number of living avirulent Pasteurella pestis organisms elicited good immunological response in guinea pigs.

Extending the original investigations carried out with white rats Downs et al (21) were able to follow the course of infection in these animals subsequent to intradermal and intraäbdominal inoculation of Bacterium tularense. It appeared that the general pathway of invasion in normal animals was through the regional and mesenteric lymph nodes into the blood stream, the organisms quickly becoming localized in two major foci, the spleen and liver. Studies of infection in immunized rats or rats recovered from infection showed that after an initial spread to the above metnioned tissues multiplication was brought to a halt and the rats

were able to rid themselves of the invading organisms quite efficiently. The organisms spread through the body with much greater rapidity in normal than in resistant rats. They also demonstrated that strains of lowered virulence invaded the rat by the same pathways as did virulent strains but their rate of spread through the animal was greatly reduced as compared to the highly virulent strains. Working with Pasteurella pestis Jawetz and Meyer (22) report almost exactly the same sequence of lymphatic and hematogenic invasion of the virulent organisms in guinea pigs and rats. They also observed that invasion by avirulent strains follows the same pattern, but at a slower rate than that of the virulent strains.

Buchele and Downs (23) determined the agglutinin titers in white rats following infection with living Bacterium tularense and after immunization with killed culture vaccines. They found that the rise in agglutinin titer closely parallels the development of immunity to tularemia. The agglutinin titer reached a peak between 7 and 14 days following vaccination and receded gradually until 99 days following vaccination the titers averaged 1:20. Immunity to challenge doses of virulent organisms persisted for as long as 99 days in vaccinated, and 114 days in recovered rats. They also were able to demonstrate that normal rats, vaccinated rats, and rats recovered from infection harbored the organisms from 33 to 46 days after infection. It is thought by some investigators that immunity to tularemia is dependent upon the continued presence of viable organisms in the animal body. Foshay (24) has isolated Bacterium tularense from the bursal fluid of humans as long as five months following infection, corresponding to three months after cessation of all disability due to the disease.

Both Downs (25) and Foshay et al (26) have emphasized the value

of vaccinating laboratory personnel engaged in tularemia research. It has been their experience that vaccination provides a fair degree of protection against tularemia. Those workers who become infected suffer relatively milder infections than do unimmunized individuals. Kadull et al (5) substantiate this viewpoint.

Observing that heat killed and formalized Bacterium tularense vaccinesccaused severe local and systemic reactions upon initial injection into apparently normal individuals, Foshay et al (26) prepared vaccines oxidized by the action of acetic acid in a sodium nitrate solution in which the organisms were suspended. They reported that antigenicity was not impaired while the toxicity of the vaccine was greatly reduced. Other refinements were made in preparing vaccines for use at Camp Detrick during the war. The human vaccine now in use on this project and at Camp Detrick was prepared by Foshay and is one that retains good immunizing qualities while the factors causing undesirable local and systemic reactions have been eliminated to a large degree.

Other vaccines for the immunization of laboratory animals have been prepared by Downs et al (6) and Coriell et al (7). These include acetone extracted vaccines, phenolized, ultraviolet irradiated and chloroform killed preparations from broth culture, and embryonated eggs. As previously noted (6 , 19) the white rat gives good immunological response to all of the vaccines, while white mice are immunized only to a very slight degree. In this regard mention should be made of Jawetz and Meyer's theory (20) that the antigenic constituent present in living avirulent Pasteurella pestis responsible for immunizing guinea pigs may be destroyed by killing the organisms. In this case mice either do not require this constituent or respond satisfactorily to extremely small amounts of the

antigen that persist in the killed vaccine.

Elbert (27) and Elbert and Gaiskii (28) report the successful immunization of guinea pigs and rabbits using cultures of lowered virulence. They do not give the LD_{50} of the strain used nor do they state what strain was used for challenge. They state that the use of dead vaccines was not successful. They also report on the use of living strains of lowered virulence as a successful means of immunizing man.

Investigations by Cromartie et al (29) have demonstrated that Bacillus anthracis produces an immunizing antigen in the tissues of rabbits which is not demonstrable when the organism is grown in ordinary culture media. Supplementing these findings is the work of Gladstone (30) who was able to isolate this same constituent from anthrax cultures containing plasma or serum. The immunizing substance was found to be a polypeptide of d-glutamic acid which constitutes the capsular material surrounding the Bacillus anthracis cell. It is tempting to visualize a parallel mechanism existing for Bacterium tularense.

While discussing labile antigenic constituents it is pertinent to mention the Vi antigen of Salmonella typhi described by Felix (31) who demonstrated its presence by agglutinin absorption tests. It appears to elicit good immunogenic response in white mice but is quite easily destroyed by the usual methods for preparing vaccines.

In order to extract the Vi antigen from Salmonella typhi without destroying or denaturing it Chambers and Flosdrof (32) described a method of sonic disintegration. The organisms were subjected to high frequency sound waves in a sonic oscillator for 45 minutes. Centrifugation removed the bacterial debris and the resulting supernatant was shown to contain antigen that precipitated Vi antibody in sera from which the H and O

antigens had been absorbed.

There are several reports in the literature concerning the treatment of tularemia with specific antiserum. Foshay (33) (34) presented the first evidence that serum treatment might be effective in treating tularemia in man. He prepared a potent anti-tularense serum from goats and demonstrated prompt improvement in 14 out of 15 tularemia patients. Treatment with antiserum resulted in a marked shortening of the period of disability and course of the disease. Francis and Felton (35) reported that the treatment of several severe cases of tularemia with convalescent human serum proved to be of no value in relieving the patients' condition. They also prepared anti-tularense serum from horses, sheep and rabbits, and could not demonstrate the presence of significant amounts of protective antibody as measured in white mice. A definite increase in the survival time of tularemic mice was observed as compared with normal controls. However, the actual survival rate was no better than that of the control animals.

The discovery of streptomycin has presented the medical sciences with another tool with which to combat tularemia. Foshay and Pasternack (36) and Foshay (37, 38) treated several tularemia patients with streptomycin and found that the clinical symptoms of headache, chills and nausea were strikingly relieved as early as two days after streptomycin therapy was begun. They compared this treatment with serum therapy and were of the opinion that it was far superior to, and would eventually replace, immune serum therapy. Heilman (39) has shown that streptomycin has a potent protective effect for white mice infected with tularemia when administered in divided doses totaling 1000 units per day for 10 days. Upon reinfection with 1000 lethal doses of Bacterium tularense the mice were

shown to have very slight immunity since almost all died as a result of the infection.

Additional studies on streptomycin therapy in tularemia were made by Chapman et al (40, 41, 42) during the war. There it was shown that monkeys, rats and mice were all protected against tularemia by streptomycin therapy if begun soon enough following infection of the animals. Mice that survived infection with Bacterium tularense as a result of streptomycin treatment, were not resistant to re-infection with these organisms. The mice were shown to harbor Bacterium tularense in the spleen for as long as 50 days unless streptomycin treatment was continued 10 to 14 days. Extended treatment with streptomycin appeared to reduce the carrier rate significantly.

MATERIALS AND METHODS

The strains of Bacterium tularense studied were from various sources and of varying degrees of virulence. A mouse titration was made before use of the strains as immunizing agents. The LD_{50} was calculated by the Reed and Muench method (43). All cultures were made on glucose cysteine blood agar (GCBA) Downs et al (44) except that Snyder's broth (45) and Mills' liquid medium (46) were employed in the preparation of the killed vaccines. The following protocol was observed as standard procedure in all mouse titrations.

A 24 hour slant culture of each of the strains was emulsified in saline and adjusted to a turbidity permitting 24 per cent light transmittance when tested in the Coleman No. 11 Universal Spectrophotometer at wave length 600. Ten-fold serial dilutions were made and plate counts and mouse titrations were carried out. Six mice were used for each dilution tested. The mice were observed for a period of 10 to 14 days. The dead mice were autopsied and spleens and heart blood cultured to recover Bacterium tularense as confirmation of infection. The range of dilutions used was determined by the results of previous titrations of these cultures. All mice were inoculated intraabdominally. Virulence titrations made immediately before these present tests show slight variations in virulence from the titrations made in 1944. With the exception of the strain "Ince" no significant changes had occurred. The Ince strain when isolated in 1944 was fully virulent and has since undergone a slight progressive decrease in virulence. The highly virulent Sm strain was used to challenge the vaccinated mice in all cases except one in which the Ince, Groves and Cruse strains were used to challenge a group of mice immunized with organisms of the Jap strain. The original source and

mouse LD_{50} of the strains used in this investigation are given in table 1.

The mice used in these experiments were approximately 20 grams in weight and were obtained from the Maple Grove Rabbitry, Springfield, Missouri. Guinea pigs of 450 grams in weight were obtained from Tumblebrook Farms, New York. Two-hundred gram hooded rats were obtained from Grant's Pet Shop, Lawrence, Kansas.

The streptomycin used in this work was Streptomycin sulfate manufactured by Merck and Company, Rahway, New Jersey.

Necessary details of technique are given under the separate experiments.

Table 1

Geographic source and mouse LD_{50} of strains of Bacterium tularense

Designation of culture	Geographic source and year of isolation	Pathologic source	Mouse LD_{50}
38	Utah 1920	Human lymph node	10^{0}
Depue	Ohio 1938	Human lymph node	10^{0}
Carr	Ohio 1944	Human lesion	10^{0}
26	Utah 1921	Human blood	$10^{-1.0}$
Chara	Japan 1931	?	$10^{-1.0}$
Max	Russia 1928	Human lymph node	$10^{-1.7}$
Russ	Russia 1928	Human lymph node	$10^{-2.0}$
Jap	Japan 1926	Human lymph node	$10^{-3.8}$
RI	Virginia 1932	Human pus	$10^{-5.93}$
Ince	Kansas 1944	Digital lesion	$10^{-7.8}$
Sm	Ohio 1941	Human ulcer	$10^{-9.5}$
Cruse	Missouri 1948		$10^{-9.5}$
Groves	Missouri 1948		$10^{-9.5}$

EXPERIMENTAL

The first experiments were conducted in an attempt to determine whether the inoculation of a sub-lethal concentration of living organisms from our stock strains of Bacterium tularense would confer any immunity on white mice.

Mouse immunization studies with the Jap strain of Bacterium tularense

A group of mice were inoculated with living organisms of the Jap strain of Bacterium tularense. The dosage was 0.5 ml of a 10^{-4} dilution of a standard suspension of the organisms. This was calculated to be just below the LD_{50} of this culture. Twenty-seven days following inoculation the mice were challenged with the following concentrations of the highly virulent Sm strain. Twelve mice received approximately 100 LD_{50}, 6 mice received 10 LD_{50}, and 6 mice received one LD_{50}. A summary of the results obtained is presented in table 2.

Since the 24 mice challenged survived and the normal mice all died these results appeared to be of significance with regard to the immunogenic properties of the Jap strain. Accordingly, attempts were made to repeat them in order to demonstrate conclusively the immunogenic nature of the Jap strain. In addition to the immunization of mice with a 10^{-4} dilution of the Jap organisms other groups were immunized with Jap dilutions at 10^{-5}, which represented approximately 20,000 organisms per mouse. It may be observed in table 3 that a high degree of immunity was elicited in the mice which was independent of the concentration of Jap organisms employed. The mortality among the mice following immunization with 20,000 Jap cells was nearly zero as compared with a 20 to 40 per cent mortality among mice receiving 200,000 organisms (Jap 10^{-4}). It is

obvious that immunization with only 20,000 Jap cells elicited good immunity while assuring a high percentage of survivors and for this reason may be preferable to the higher concentration for immunity studies. A total of 304 mice were vaccinated with the Jap organisms in the experiments summarized in table 2 and 3. Only 19 mice died as a result of infection with Bacterium tularense following challenge with the highly virulent Sm strain, giving a mortality of 6.5 per cent as compared with a mortality of 100 per cent observed in the normal control mice.

Since vaccination with living Jap organisms protected white mice against the highly virulent Sm strain of Bacterium tularense it was of interest to determine whether this immunity was effective against other highly virulent strains. To determine this, a group of mice were inoculated with approximately 2.8×10^4 Jap organisms and were challenged 14 days later with 10,000 LD_{50}, 1,000 LD_{50}, and 100 LD_{50} of the Cruse, Groves and Ince strains. Ten mice were challenged with 100 LD_{50} of the Sm strain for comparison. Four groups of six normal mice served as controls for the four challenge organisms. They each received an infecting dose of 10 LD_{50}. The results are presented in table 4. It may be observed that immunization with living Jap organisms elicits a high degree of immunity to more than one fully virulent strain of Bacterium tularense. In their work on the immunization of white mice Coriell et al (7) reported that the few animals which survived a challenge dose of Sm following immunization showed little resistance to rechallenge with the virulent strain. To determine whether mice immunized with living Jap were resistant to rechallenge with highly virulent Sm the following experiment was performed. Twenty-five mice were immunized with the Jap strain and challenged

Table 2

Resistance of mice to challenge with virulent

Bacterium tularense after inoculation with the Jap strain

Strain	Original inoculation	Test dose of virulent organisms (Sm)		
		1 LD_{50}	10 LD_{50}	100 LD_{50}
Jap	2.6×10^5	0/6*	0/6	0/12
Normal mice		6/6	6/6	

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Table 3

Resistance of mice to challenge with virulent

Bacterium tularense after inoculation with the Jap strain

Strain	Original inoculation	Test dose of virulent organisms (Sm)			
		10 LD_{50}	100 LD_{50}	1000 LD_{50}	10,000 LD_{50}
Jap	1.3×10^5		1/10*	2/10	1/10
Jap	1.3×10^4	0/10	0/10	3/10	3/10
Jap	3.7×10^5		0/7	0/10	3/10
Jap	2.0×10^5	0/10	0/10	0/10	1/10
Jap	2.9×10^4		1/50	3/50	1/43
Normal mice		6/6	28/28		

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Table 4

Resistance of mice to challenge with more than one virulent strain of Bacterium tularense after inoculation with the Jap strain

Original Inoculation	Challenge strain	Test dose of virulent organisms 10 LD_{50}	100 LD_{50}	1000 LD_{50}	10,000 LD_{50}
2.8×10^4	Sm		0/20*		
	Control mice	6/6			
2.8×10^4	Groves		0/20	1/20	2/20
	Control mice	6/6			
2.8×10^4	Cruse		1/20	3/20	1/20
	Control mice	6/6			
2.8×10^4	Ince		0/10	0/10	0/10
	Control mice	5/6			

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

with 100 LD_{50} of the Sm strain 28 days later, then rechallenged 30 days after challenge. The results are shown in table 5.

It may be observed that the mice exhibited a high degree of resistance to the second inoculation with the Sm strain. These results are in direct contrast to those obtained by Coriell et al (7) using mice immunized with killed culture vaccines.

Discovery of the immunogenic properties of living Jap organisms encouraged the investigation of other strains of lowered virulence. The completely avirulent strain 38 was tested for immunogenic properties. Three groups of white mice were inoculated with living 38 organisms as follows: group 1 received 0.5 ml of a 10^0 suspension representing approximately 3.0×10^9 cells; group 2 received 3.0×10^9 cells on each of two alternate days; group 3 received 3.0×10^9 organisms on each of three alternate days. Consequently group 2 received twice, and group 3 received three times the concentration of cells given to the mice in group 1.

Twenty-five days following the first injection the mice were challenged with 100 LD_{50}, 10 LD_{50}, and 1 LD_{50} of the Sm strain. The results are summarized in table 6. It may be observed that strain 38 did not elicit a good immunological response in white mice. Even the mice in group 3 which received three times the number of organisms given the mice in group 1 were not well protected. On the other hand, a minimum of 200 living Jap cells has been shown to provide good protection against highly virulent Bacterium tularense, (table 10).

It appeared that in the case of the Jap and 38 strains there was a decided difference in immunizing ability. In view of this, it was decided to investigate several other strains of lowered virulence in order

Table 5

Resistance of Jap-immunized mice to rechallenge with the Sm strain of Bacterium tularense

Inoculation	Test dose of virulent organisms (Sm) 1 LD_{50}	10 LD_{50}	100 LD_{50}
Challenge			0/25*
Rechallenge			2/25
Normal mice	3/6	6/6	

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Table 6

Resistance of mice to challenge with virulent Bacterium tularense after inoculation with organisms of strain 38

Group	Original inoculation	Test dose of virulent organisms (Sm) 1 LD_{50}	10 LD_{50}	100 LD_{50}
1	3.0×10^9	3/6*	6/6	5/6
2	6.0×10^9 (in two doses)	3/6	5/6	6/6
3	9.0×10^9 (in three doses)	1/6	5/6	3/6
Normal mice		3/6	6/6	

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

to evaluate their immunogenic properties, and to determine their relationship in this respect to that of Jap and 38. The following cultures were tested for immunogenic properties in white mice: Ohara, 26, Max, and Russ. Groups of mice were inoculated with doses just below the LD_{50} of these strains and were challenged from 10 to 28 days later. The results are summarized in table 7. It may be observed that only the Russ strain elicits an immunological response comparable to that of the Jap strain of Bacterium tularense. There appears to be little immunogenic difference among the strains Ohara, 26, and Max. However, a comparison of table 7 with table 5 serves to stress the fact that these strains are more active immunologically than is the avirulent strain 38.

Table 7

Resistance of mice to challenge with virulent Bacterium tularense after inoculation with various strains of lowered virulence

Strain	Original inoculation	LD_{50}	test dose of virulent organisms (Sm) 1 LD_{50}	10 LD_{50}	100 LD_{50}	1000 LD_{50}	10,000 LD_{50}
Chara	1.1×10^7	$10^{-1.0}$		4/10*	5/10	7/10	7/10
Chara	5.9×10^7	$10^{-1.0}$		4/10	3/10	6/10	5/10
26	approximately 1.5×10^7	$10^{-1.0}$		0.10	6/10	8/10	8/10
Max	approximately 1.5×10^7	$10^{-1.7}$		6/10	5/10	7/10	6/10
Russ	1.7×10^6	$10^{-2.0}$		1/10	0/10	1/10m	3/10
Russ	3.3×10^6	$10^{-2.0}$			0/10	0/10	0/10
Normal mice			22/30	28/30			

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

The duration of immunity to Bacterium tularense in white mice vaccinated with living Jap organisms

Since the Jap strain of Bacterium tularense was highly immunogenic it was of interest to determine how long mice remained immune following inoculation with living organisms of this strain. Forty mice were infected with 2.7×10^5 Jap organisms and 10 mice of this group were challenged at 30 day intervals for four months with 100 LD_{50} of virulent Sm. Ten normal control mice were infected at each time interval. The results are presented in table 8. It is seen that the mice retained good immunity as long as four months following vaccination.

The time of appearance of immunity following the vaccination of white with the Jap strain of Bacterium tularense

The following experiment was designed to determine the time necessary to produce immunity in white mice inoculated with the Jap strain of Bacterium tularense. A group of 50 mice received 0.5 ml of a Jap suspension containing 1.6×10^4 organisms. Ten mice from the group were challenged each day for 5 days with 100 LD_{50} of the Sm strain. The results are given in table 9. The results show that immunity appears as early as 72 hours following an inoculation of the Jap strain.

Development of immunity in white mice after the inoculation of decreasing doses of the Jap strain of Bacterium tularense

Since the Jap strain had been shown to be highly immunogenic

Table 8

Resistance of mice to challenge with virulent *Bacterium tularense* over a period of four months after inoculation with the Jap strain

Time of challenge following inoculation	Test dose of virulent organisms (Sm) 100 LD_{50}	
	Jap inoculated mice	Normal control mice
1 month	0/10*	10/10
2 months	1/10	10/10
3 months	1/10	10/10
4 months	1/10	10/10

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Table 9

Appearance of immunity in white mice

following inoculation with living Jap strain

Days after inoculation of Jap strain	Test dose of virulent Sm, 100 LD_{50}	
	Jap inoculated mice	Normal control mice
1	10/10*	10/10
2	7/10	10/10
3	3/10	10/10
4	1/10	10/10
5	1/10	10/10

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

in the living state it was considered desirable to determine how small an initial dose of the living Jap cells would immunize mice. Sixty mice were divided into six groups of 10 each. The six groups were inoculated with decimally decreasing numbers of Jap organisms from 200,000 to 2 as shown in table 10. These mice were challenged 28 days later with 100 LD_{50} of the virulent Sm strain of Bacterium tularense. It would appear that from 200 to 200,000 cells as the initial dose served to confer a solid immunity to infection. Less than this number tended to give less immunity. As few as 20 Jap cells as an initial inoculation were sufficient to elicit a fair degree of immunity in the white mouse against virulent strains.

Comparative studies of the immunity produced in white mice by the Jap, Russ and Carr strains of Bacterium tularense

The widely varying results obtained using strains of lowered virulence indicated that their immunogenic properties might be related to the size of the original inoculum administered to the mice. It was known that as few as 200 living Jap organisms elicited a good immunity. Large numbers of living Russ and Carr organisms elicited good immunity in mice but it was not known how few of these cells would do so. The highly immunogenic properties of the Carr strain had been determined by other workers in this laboratory. To determine the properties of these strains using immunizing doses containing approximately the same number of organisms, three groups of 30 mice were inoculated with 0.5 ml of a 10^{-5} dilution of a standard suspension of the Jap, Russ and Carr organisms, respectively. Thirteen days later 10 mice in each group were challenged

Table 10

Immunity developing after the inoculation of decreasing doses of the Jap strain of Bacterium tularense

Dilution of suspension 0.5 ml intraäbdominally	Number of organisms per mouse	Test dose of virulent organisms (Sm) 100 LD_{50} *
10^{-4}	200,000	0/10 **
10^{-5}	20,000	0/10
10^{-6}	2,000	0/10
10^{-7}	200	1/10
10^{-8}	20	2/10
10^{-9}	2	9/10

* Infection with Sm 28 days after inoculation with the Jap strain.

** Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

with 1,000, 100, and 10 LD_{50} of the virulent Sm strain of Bacterium tularense. The results of this experiment are presented in table 11. It may be observed that the Jap strain elicits a much greater degree of immunity in mice than do the other two strains when a relatively low concentration of organisms is used for vaccination.

The disposal of virulent Bacterium tularense in mice immunized with living Jap organisms

The majority of mice immunized with living Jap organisms and other immunogenic strains of lowered virulence and subsequently challenged with virulent Bacterium tularense not only survived, but also failed to exhibit any signs of illness such as roughening of the coat, lethargy and anorexia. This indicated that the virulent organisms were disposed of very rapidly following inoculation into the immune mice. To determine how rapidly the organisms were killed a group of 15 mice was inoculated with a sub-lethal dose of living Jap strain and 27 days later each was challenged with 100 LD_{50} of virulent organisms (Sm). On the 4th, 9th, and 15th day after challenge 5 mice were killed and autopsied. The spleens were macerated individually in 2 ml of saline. Five-tenths ml of each spleen suspension was inoculated into each of four mice. It was assumed that a very small number of virulent organisms remaining in the spleen could be detected since 1 to 4 cells are sufficient to cause the death of a normal mouse. All mice that died were autopsied and spleen impression cultures were made for the recovery of virulent Bacterium tularense. Table 12 shows that as soon as 4 days following challenge the mice had disposed of the virulent organisms quite efficiently. Fourteen days after challenge one mouse appeared to be infected with the

Table 11

Resistance of white mice to challenge with virulent Bacterium tularense following inoculation with approximately 2.0×10^{4} Jap, Russ or Carr organisms

Strain	Original inoculation	LD_{50}	Test dose of virulent organisms (Sm) 10 LD	100 LD_{50}	1000 LD_{50}
Jap	2.0×10^{4}	$10^{-3.0}$	9/10*	0/10	1/10
Russ	2.0×10^{4}	$10^{-2.0}$	4/10	1/10	8/10
Carr	2.0×10^{4}	10^{0}	5/10	5/10	7/10
Normal mice			10/10		

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Table 12

Rate of disposal of virulent

Bacterium tularense by Jap-immunized mice

Days after Jap inoculation	Mice challenged with 100 LD	Mice inoculated with spleen suspensions from challenged mice
4	1	0/4*
	2	0/4
	3	0/4
	4	0/4
	5	0/4
9	1	4/4
	2	0/4
	3	0/4
	4	1/4**
	5	1/4**
14	1	2/4**
	2	0/4
	3	4/4
	4	0/4
	5	0/4

* Mortality ratio = $\frac{\text{Dead}}{\text{tested}}$

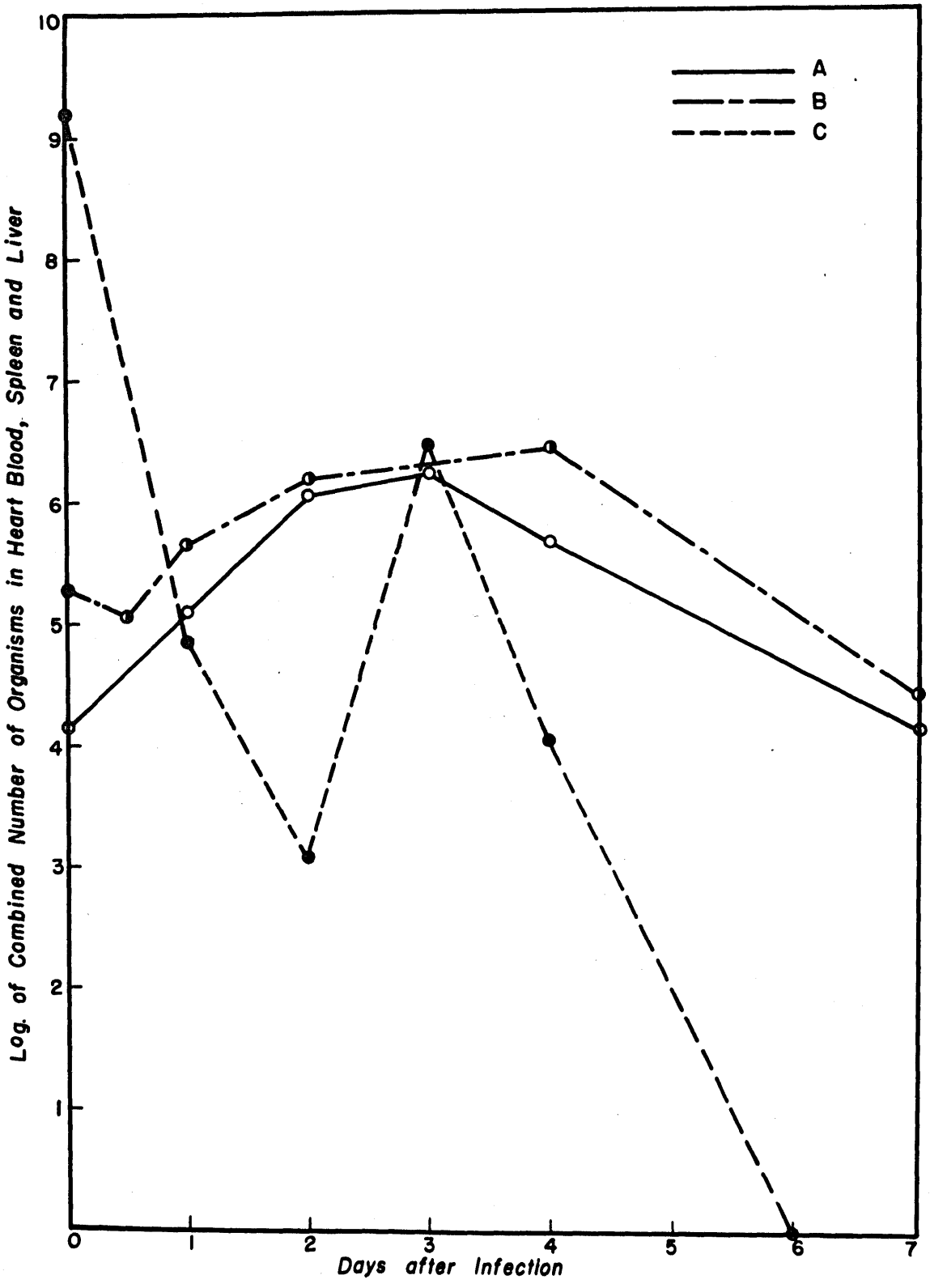
** Bacterium tularense not recovered from spleen impression

virulent Sm strain. This is not inconsistent with previous results which have shown that the immunity of any given group of Jap inoculated mice is not always complete, since a small percentage of deaths due to Bacterium tularense may occur in the group following challenge. Four mice died apparently from non-specific causes and virulent Sm organisms could not be recovered from impression cultures on GCBA.

The multiplication in white mice of strains of Bacterium tularense of lowered virulence

Since living cells of the Jap strain had been shown to be highly immunogenic for mice, it was of interest to determine the rate of multiplication of the Jap organisms and to consider this as a factor in the production of immunity in white mice. Two groups of mice were inoculated with (a) 14,000 organisms and (b) 220,000 organisms intraabdominally. A third group of mice (c) received 2.1×10^9 organisms of the "38" strain. Five mice from group "a" were sacrificed and autopsied 12 hours, 1, 2, 4, and 7 days after infection. Appropriate decimal dilutions of the heart blood were prepared and plate counts were made on GCBA. The spleens and livers were individually macerated in 2 and 10 ml of saline respectively. Serial ten-fold dilutions of the suspensions were made and plate counts were done as above. The logarithm of the average of the combined number of organisms in the spleen, liver and heart blood on the days the mice were killed is presented in figure 1. Five mice from group (b) were sacrificed 1, 2, 3, 4, and 7 days after infection, and plate counts of the heart blood, liver and spleen were done as described above. Five mice from group (c) were treated in a similar manner on the 1st, 2nd, 3rd, 4th, and 6th day following inoculation with strain 38. These

Fig. 1 The multiplication of the Jap and 38 strains of Bacterium tularense in white mice after infection using (a) 1.4×10^4, (b) 2.2×10^5 Jap organisms and (c) 2.1×10^9 organisms of strain 38, intraabdominally. The number of organisms is expressed as the log of the combined number in the heart blood, spleen and liver.


A
B
C
Log. of Combined Number of Organisms in Heart Blood, Spleen and Liver
Days after Infection
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7

results are also presented in figure 1. It is quite obvious that the organisms of the Jap strain multiplied to a great extent following their entrance into the animal body. During the course of the experiments it was possible to recover much greater concentrations of Jap organisms from the tissues of the infected mice than were present in the original inoculations. On the other hand, organisms of the 38 strain were recovered in decreasing concentrations until it was not possible to demonstrate the presence of these organisms in the mice 6 days after inoculation. While this does not constitute proof that the 38 strain did not multiply at all in mice it does indicate that this strain is not able to establish itself in these animals. The difference in size of the inoculum in groups (a) and (b) appeared to have little influence on the ultimate number of cells that could be recovered from the mice.

The survival of the Jap strain of Bacterium tularense in white mice

While it was apparent that living Jap organisms multiplied freely in mice and could be recovered in large numbers for several days following the inoculation of relatively small concentrations of cells, it was not known how long the organisms persisted in the animal body. To determine this, 21 mice were inoculated with 3.0×10^4 Jap organisms. Five mice were sacrificed on the 10th day after inoculation and four mice were killed on the 14th, 16th, 18th, 20th and 30th day. Spleen counts were made on each mouse as previously described. On the 10th day two mice did not harbor Jap organisms. The spleen counts of the three remaining mice showed 30, 20,000 and 86 Jap organisms, respectively. On the 14th day one mouse harbored 50 Jap organisms in the spleen. The

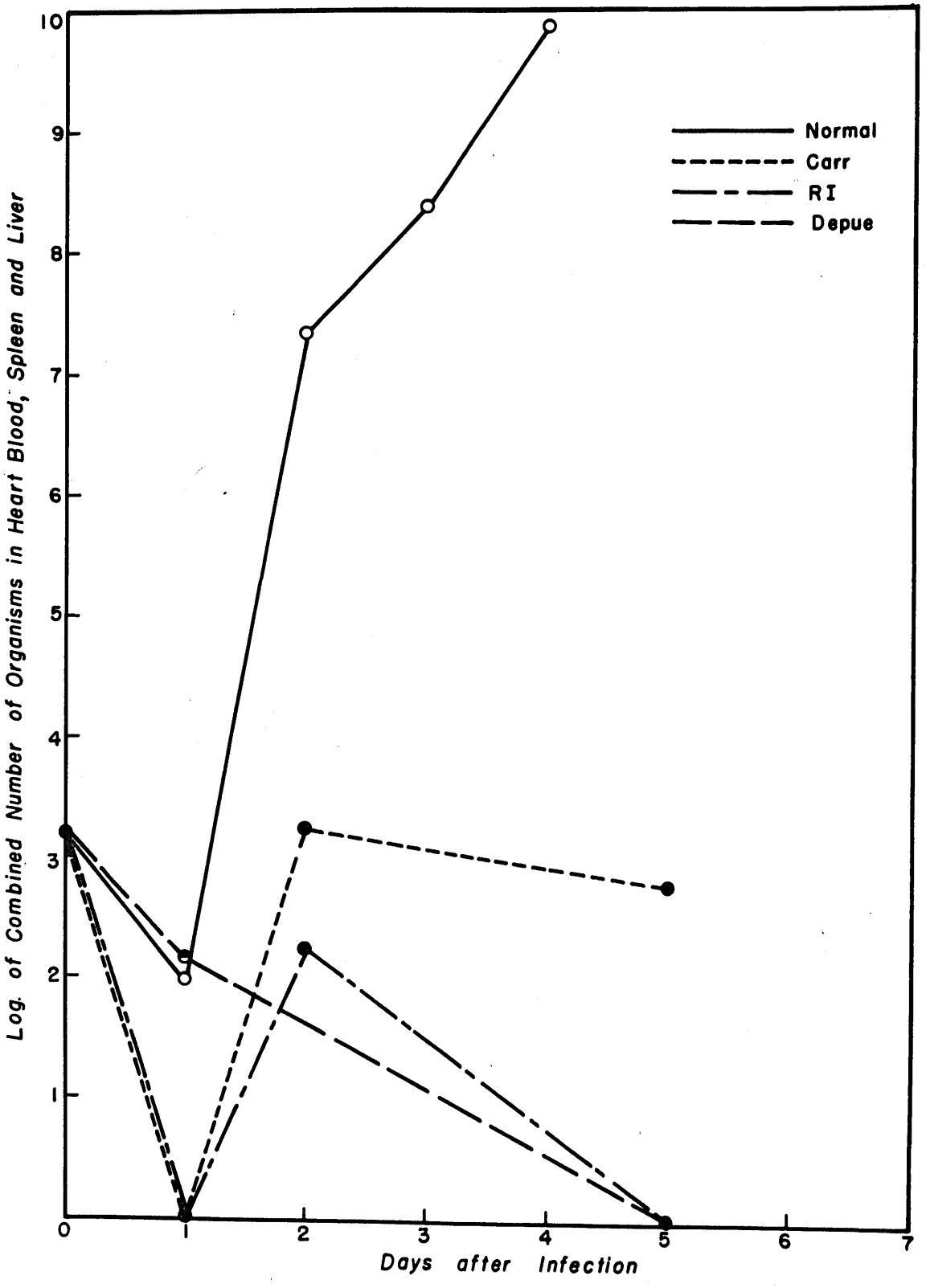
other three mice were negative. On the 16th day one mouse was shown to be harboring 680 organisms in the spleen. The other mice were free from infection. On the 18th, 20th, and 30th day all mice were shown to be free from infection with the Jap strain of Bacterium tularense.

It is apparent that the Jap organisms do not persist for more than two or three weeks following infection, as determined by spleen counts.

The disposal of virulent Bacterium tularense in mice vaccinated with 3 strains of lowered virulence

In this experiment groups of mice were inoculated with sub-lethal doses of the following strains of lowered virulence: Carr, RI, and Depue. After 27 days a control group and the immunized mice were challenged with 1,000 LD_{50} of the virulent Sm strain. The progress of the infection was followed as before by quantitative studies. Four of the immune mice and one control mouse were sacrificed at 24, 48, 72 hours and on the 5th day after infection. Figure 2 shows graphically that the immune mice were able to limit the multiplication of the virulent organisms much more effectively than the normal mice. All of the normal control mice were dead by the 5th day whereas the immune mice, as shown by counts, were rapidly ridding themselves of the organisms. The remaining immune mice survived until 15 to 21 days after inoculation when they were killed to detect possible carriers. Of the eight mice sacrificed 15 days after challange three were carriers. Only two carriers were found among the five mice sacrificed 21 days after challenge.

Fig. 2 Quantitative studies on mice recovered from a sub-lethal dose of organisms of lowered virulence and challenged with 1.6×10^3 organisms of a virulent strain (Sm). The number of organisms is expressed as the log of the combined number in the heart blood, spleen and liver.


Normal
Carr
RI
Depue
Log. of Combined Number of Organisms in Heart Blood, Spleen and Liver
Days after Infection
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
9
10

A comparison of the immunogenic properties of strains of varying virulence with their ability to multiply in white mice

The results thus far obtained indicated that the immunogenic properties of strains of lowered virulence might be directly influenced by their ability to multiply in white mice. To determine this, four strains were selected on the basis of their high immunogenic activity and their difference in virulence. White mice were infected with graded concentrations of each of the cultures followed by challenge with graded concentrations of the Sm strain.

Strains RI, Jap, Russ and Depue were selected for study. It may be observed in table 1 that the LD_{50} of these strains ranged from $10^{-5.9}$ in the case of RI to 10^{0} in the case of the Depue strain. Groups of mice were then infected with the following concentrations of organisms from the four strains.

Strain RI

Group 1 1.4×10^{3} organisms

Group 2 1.4×10^{2} organisms

Group 3 1.4×10^{1} organisms

Strain Jap

Group 1 2.6×10^{4} organisms

Group 2 2.6×10^{3} organisms

Group 3 2.6×10^{2} organisms

Group 4 2.6×10^{1} organisms

Strain Russ

Group 1 5.5×10^{5} organisms

Group 2 5.5×10^{4} organisms

Group 3 5.5×10^3 organisms

Group 4 5.5×10^2 organisms

Group 5 5.5×10^1 organisms

Strain Depue

Group 1 7.0×10^7 organisms

Group 2 7.0×10^6 organisms

Group 3 7.0×10^5 organisms

Group 4 7.0×10^4 organisms

Group 5 7.0×10^3 organisms

Group 6 7.0×10^2 organisms

Group 7 7.0×10^1 organisms

On the 1st, 2nd, 3rd, 4th, 5th, 7th, and 9th day following inoculation, mice from each group were sacrificed and spleen counts were made as previously described. The remainder were saved for a study of the survival rate following challenge. The groups saved for survival studies were sub-divided into 3 groups each and the mice were challenged 11 days after inoculation with 1000, 100 and 10 LD_{50} of the Sm strain, respectively.

The course of infection with the strains of lowered virulence is shown graphically in figures 3, 4, 5, and 6. The survival rates of the mice following challenge with the Sm strain are presented in tables 13 and 14.

An inspection of the figures shows that the ability of the strains to multiply in the animals can be correlated directly with their virulence. Also, it may be observed that the degree of immunity produced by the strains is in direct relationship to their ability to multiply in the mice.

All of the groups inoculated with the Jap strain appeared to have

Fig. 3 The multiplication of the RI strain of Bacterium tularense in white mice using (a) 1.4×10^3, (b) 1.4×10^2 and (c) 1.4×10^1 organisms intraabdominally. The number of organisms is expressed as the log of the number in the spleen.

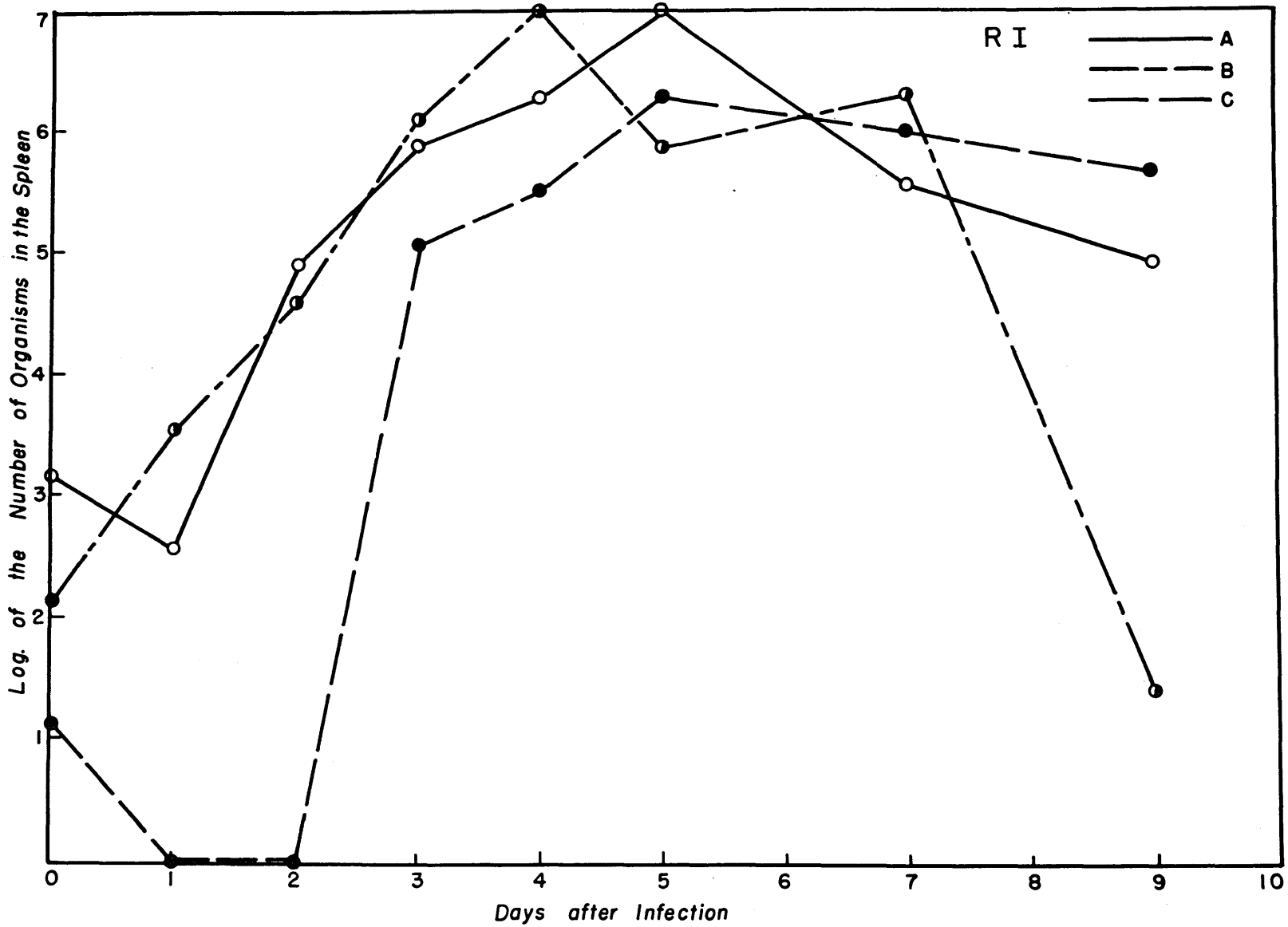

RI
A
B
C
Log. of the Number of Organisms in the Spleen
Days after Infection
0
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
6
7

Fig. 4 The multiplication of the Jap strain of Bacterium tularense.in white mice using (a) 2.6×10^4, (b) 2.6×10^3, (c) 2.6×10^2 and (d) 2.6×10^1 organisms intra-abdominally. The number of organisms is expressed as the log of the number in the spleen.

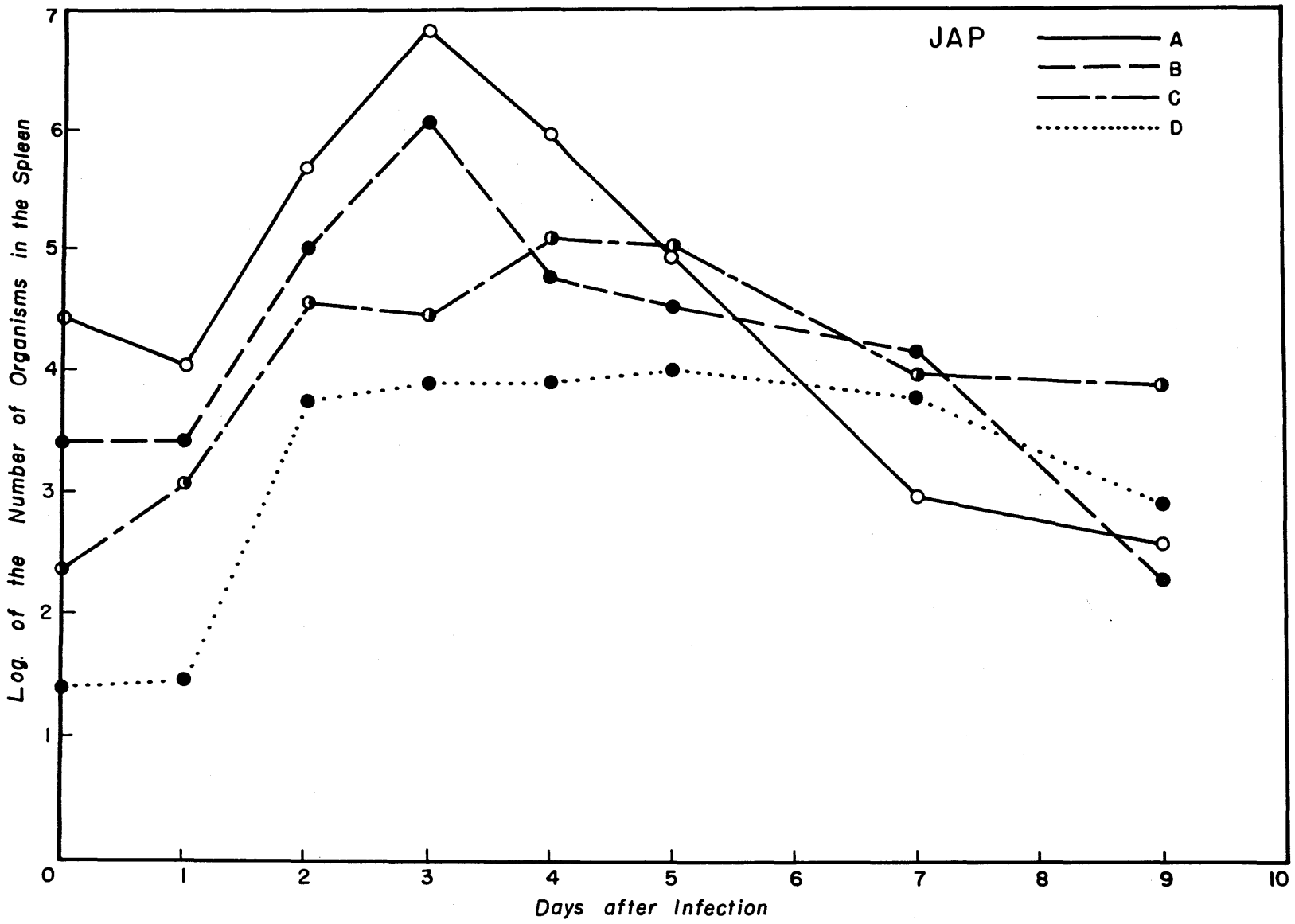

JAP
A
B
C
D
Log. of the Number of Organisms in the Spleen
Days after Infection
0
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
6
7

Fig. 5 The multiplication of the Russ strain of Bacterium tularense in white mice using (a) 5.5×10^5, (b) 5.5×10^4, (c) 5.5×10^3, (d) 5.5×10^2, and (e) 5.5×10^1 organisms intraabdominally. The number of organisms is expressed as the log of the number in the spleen.

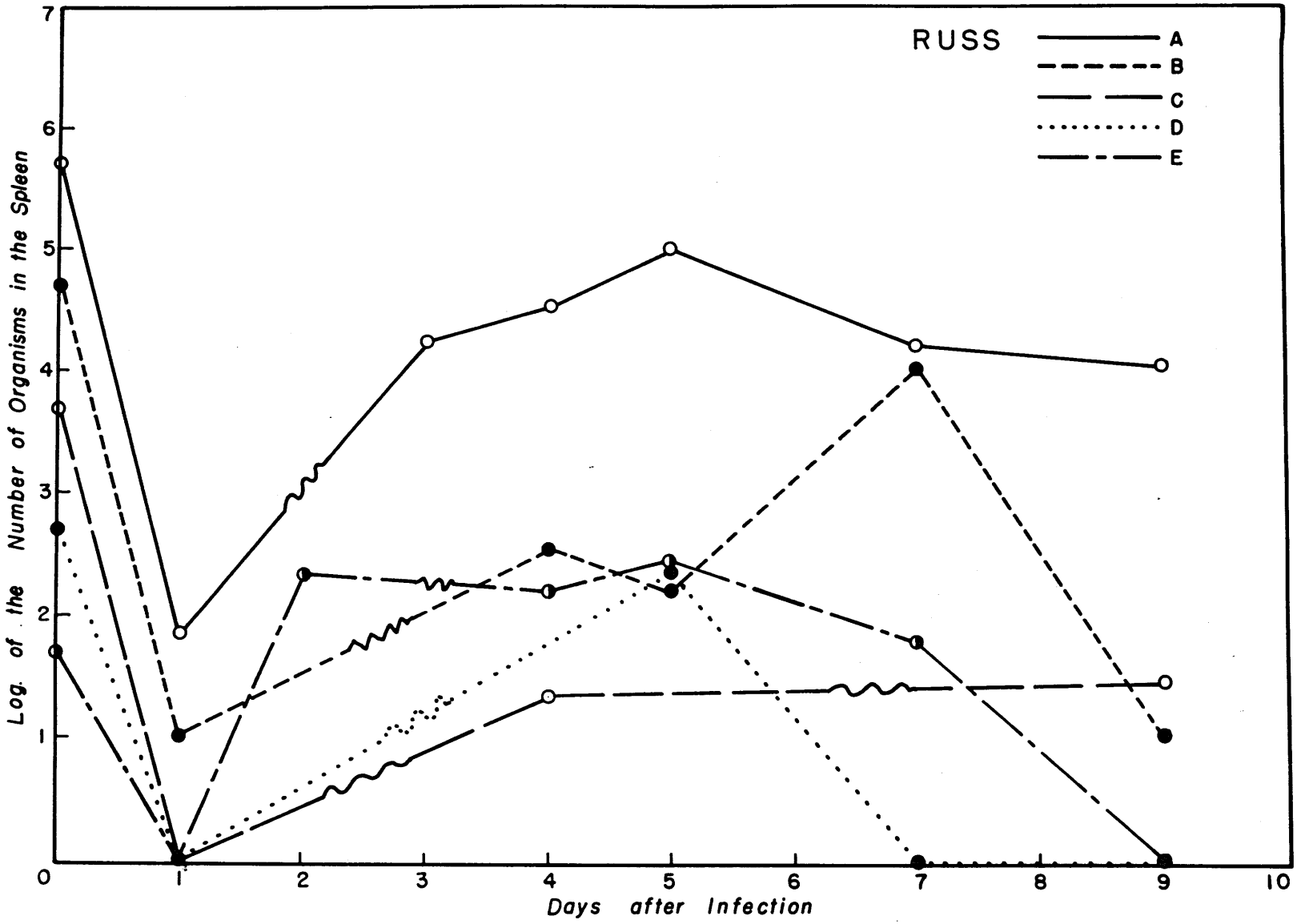

RUSS
A
B
C
D
E
Log. of the Number of Organisms in the Spleen
Days after Infection
0
1
2
3
4
5
6
7
8
9
10

Fig. 6 The multiplication of the Depue strain of Bacterium tularense in white mice using (a) 7.0×10^7, (b) 7.0×10^6, (c) 7.0×10^5, (d) 7.0×10^4, (e) 7.0×10^3, (f) 7.0×10^2, and (g) 7.0×10^1 organisms intraabdominally. The number of organisms is expressed as the log of the number in the spleen.

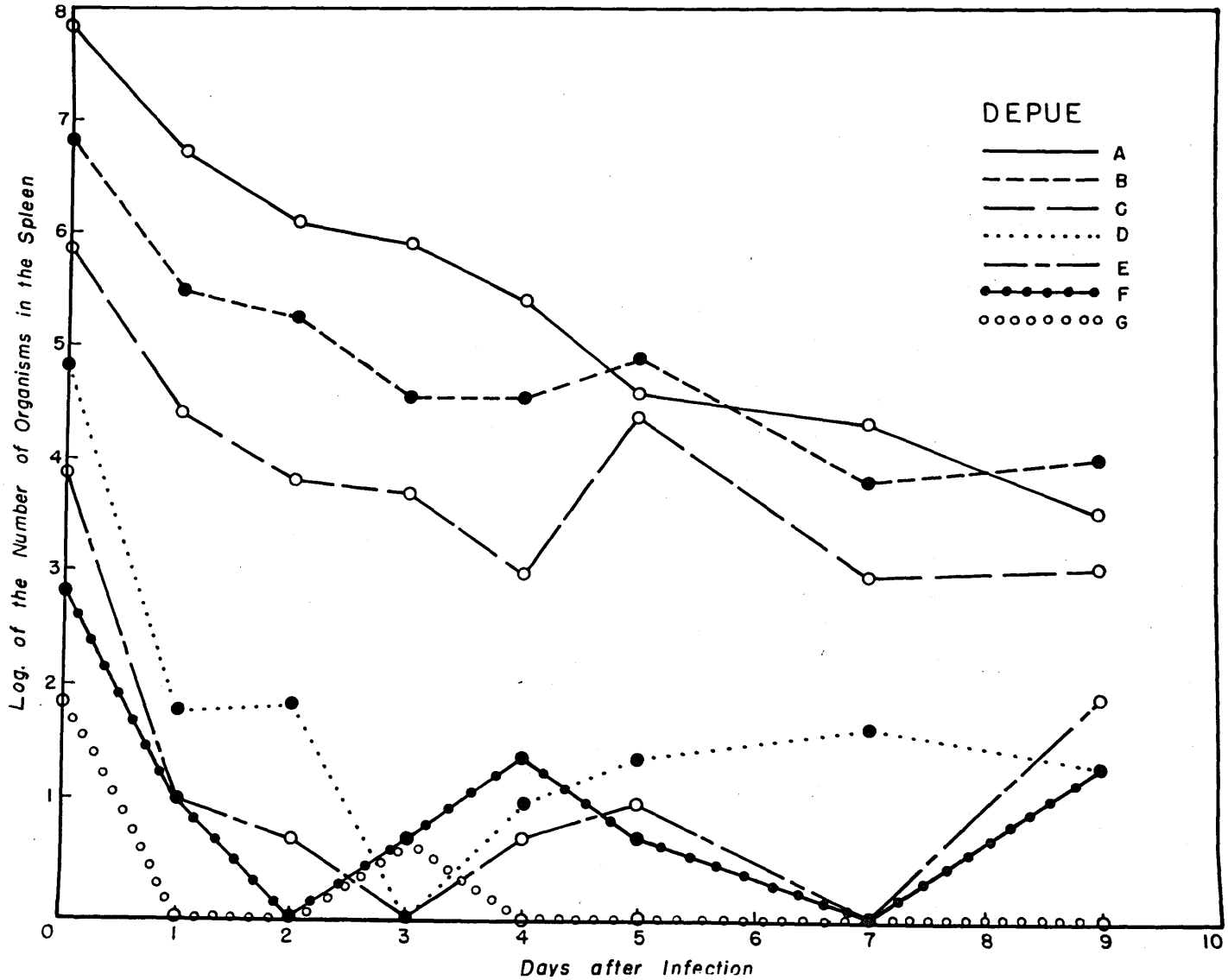

DEPUE
A
B
C
D
E
F
G
Log. of the Number of Organisms in the Spleen
Days after Infection
0
1
2
3
4
5
6
7
8
9
10

Table 13

Resistance of white mice to challenge with virulent Bacterium tularense following inoculation with the RI and Jap strains

Strain	Original inoculation	Test dose of virulent organisms 10 LD_{50}	100 LD_{50}	1000 LD_{50}	Total Deaths	Per cent survival
RI	1.4×10^3	-	0/8*	0/8	0/16	100
RI	1.4×10^2	3/10	1/10	2/5	6/25	76
RI	1.4×10^1	6/10	6/10	3/10	15/30	50
Normal mice		10/10	10/10	10/10	30/30	0
Jap	2.6×10^4	1/11	0/11	0/14	1/36	97.2
Jap	2.6×10^3	3/11	3/10	1/17	7/38	81.5
Jap	2.6×10^2	0/14	0/11	0/11	0/36	100
Jap	2.6×10^1	4/14	5/11	1/11	10/36	72.2
Normal mice		10/10	8/10	10/10	28/30	6.6

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Table 14

Resistance of white mice to challenge with virulent Bacterium tularense following inoculation with the Russ and Depue strains

Strain	Original inoculation	Test dose of virulent organisms LD_{50}	100 LD_{50}	1000 LD_{50}	Total deaths	Per cent survival
Russ	5.5×10^5	2/11	1/11	3/10	6/32	81.2
Russ	5.5×10^4	5/11	6/11	11/14	22/36	38.8
Russ	5.5×10^3	8/11	11/11	10/10	29/32	9.3
Russ	5.5×10^2	10/11	10/11	11/13	31/35	11.4
Russ	5.5×10^1	2/12	10/11	10/12	22/35	37.1
Normal mice		10/10	8/10	10/10	28/30	6.6
Depue	7.0×10^7	1/10	1/10	3/15	5/35	85.7
Depue	7.0×10^6	1/10	5/10	9/15	15/35	57.1
Depue	7.0×10^5	6/10	7/10	14/16	27/36	25.0
Depue	7.0×10^4	9/10	10/10	15/15	34/35	2.8
Depue	7.0×10^3	8/10	10/10	16/16	34/36	5.5
Depue	7.0×10^2	9/10	10/10	15/15	34/35	2.8
Depue	7.0×10^1	9/10	10/10	9/9	28/29	3.4
Normal controls		10/10	10/10	10/10	30/30	0

a relatively high degree of immunity. The same holds true for the mice inoculated with RI. On the other hand, only the group 1 Russ mice were highly resistant to infection with the Sm strain. Significantly lower resistance was noted for the other four Russ groups. Similar results may be observed in the case of the mice inoculated with Depue.

The production of agglutinins by white mice infected with strains of Bacterium tularense of lowered virulence

An attempt was made to determine the agglutinin titers produced by the inoculation of living organisms of the Jap, Russ, 26 and 38 strains into white mice. Four groups of mice each received a sub-lethal inoculation with one of the above strains. Five mice from each group were killed by bleeding from the heart on the 2nd, 7th, 12th, 17th and 22nd day after inoculation. Agglutination tests were performed on each blood sample as follows: A 1:10 dilution of serum was made and from this serial two-fold dilutions were prepared in 0.2 ml of 0.85 per cent saline. To the dilutions was added 0.2 ml of a standard suspension of formalized agglutination antigen prepared with organisms of strain 38. The tubes were then placed in a 37°C water bath for 16 to 18 hours, and read. The dilutions used were 1:20, 1:40, 1:80 and 1:160. This experiment was repeated in nearly the same manner, except that only three mice from each group were bled on the 6th, 8th, 10th, 12th, 14th, 16th, and 18th day. The average agglutinin titer was determined for each group and is shown graphically in figures 7, 8, 9 and 10. It may be observed that the Jap and Russ strains produced higher agglutinin titers than did strains 26 and 38. Mice vaccinated with strain 38 showed the presence of agglutinins only on the 7th and 8th day. The agglutinin titer produced by each strain

Fig. 7 Development of agglutinins in mice following infection with the Jap strain of *Bacterium tularense*.

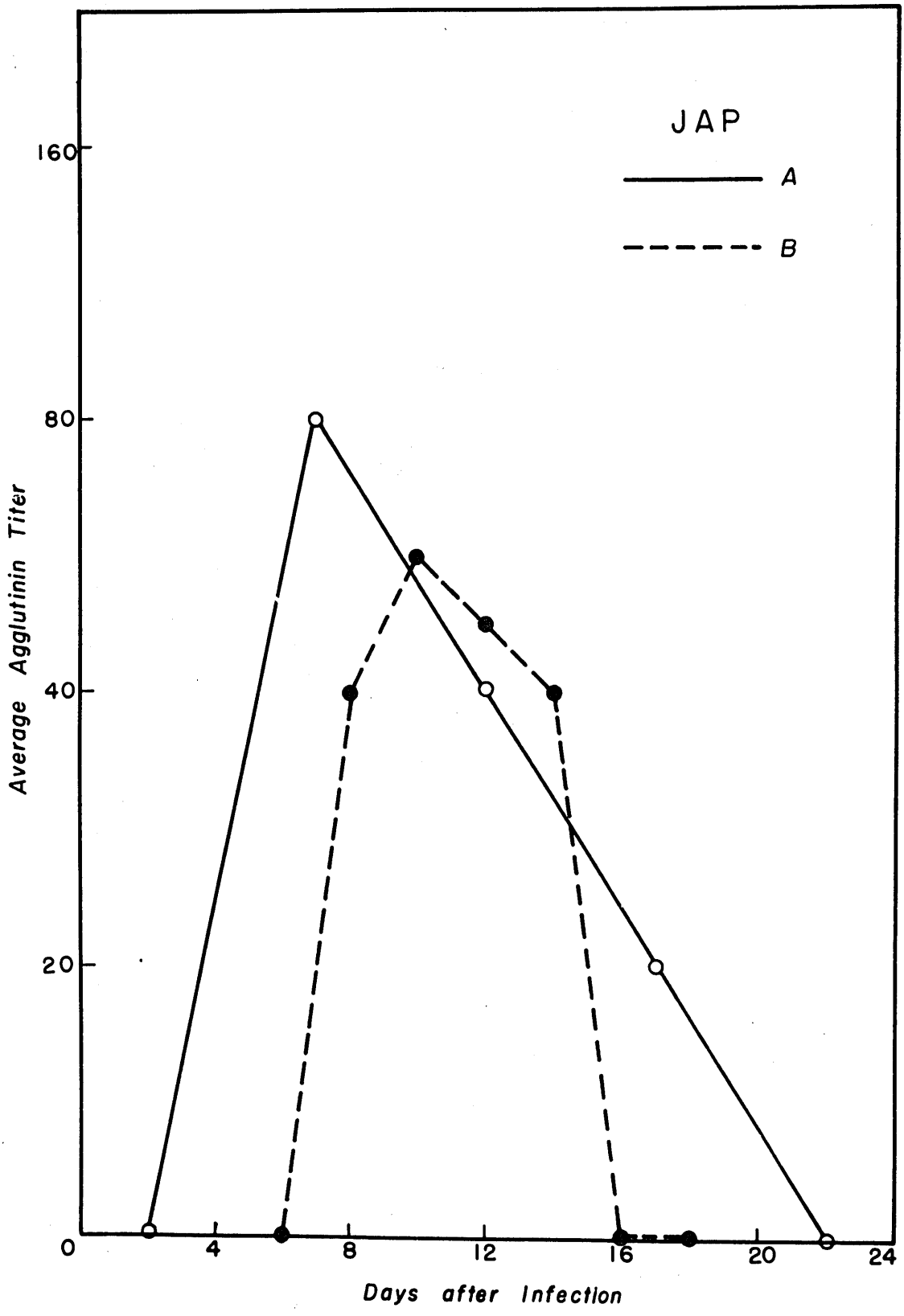

JAP
A
B
160
80
40
20
0
Average Agglutinin Titer
4
8
12
16
20
24
Days after Infection

Fig. 8 Development of agglutinins in mice following infection with the Russ strain of *Bacterium tularense*.

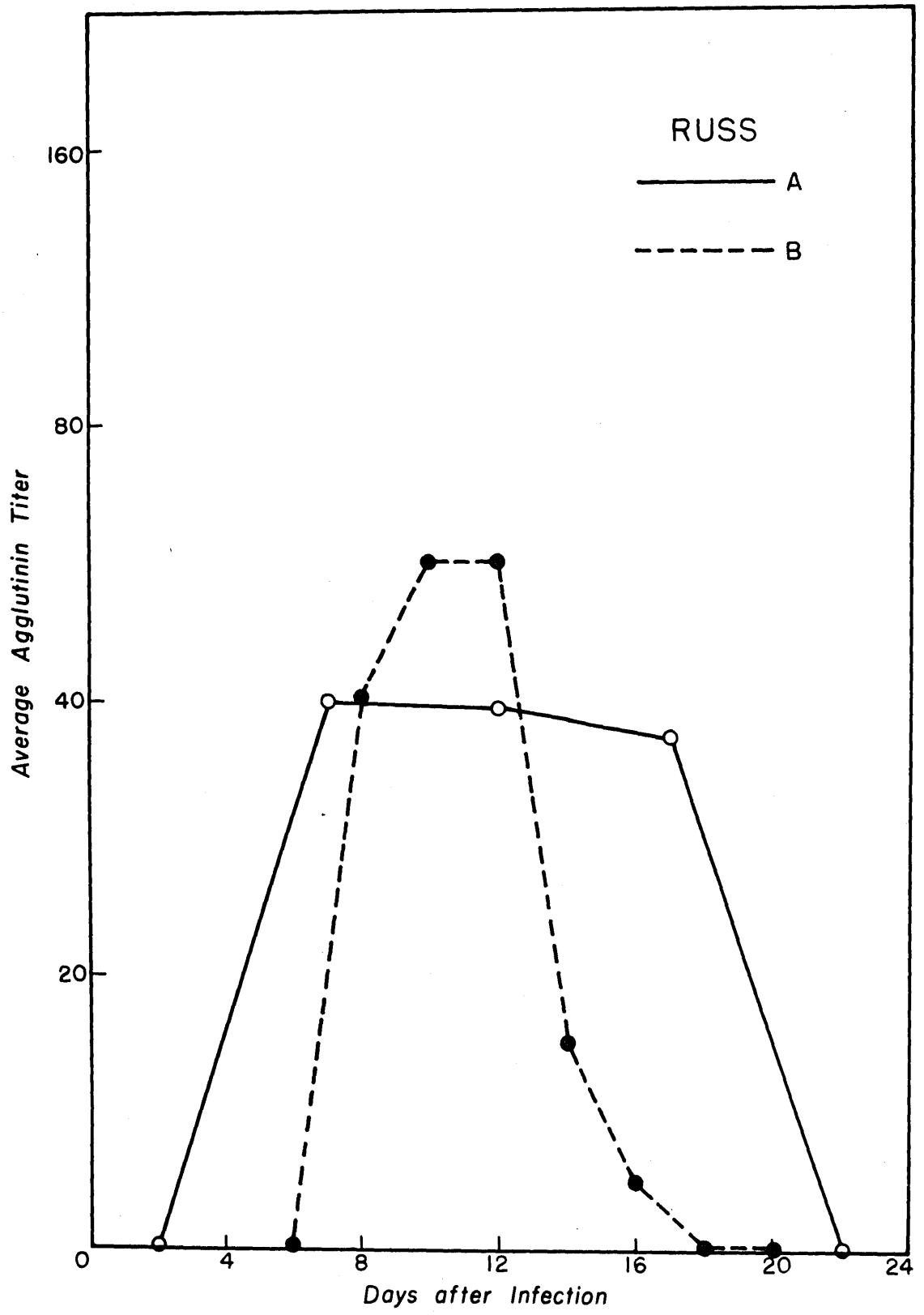

RUSS
A
B
160
80
40
20
0
4
8
12
16
20
24
Average Agglutinin Titer
Days after Infection

Fig. 9 Development of agglutinins in mice following infection with the 26 strain of *Bacterium tularense*.

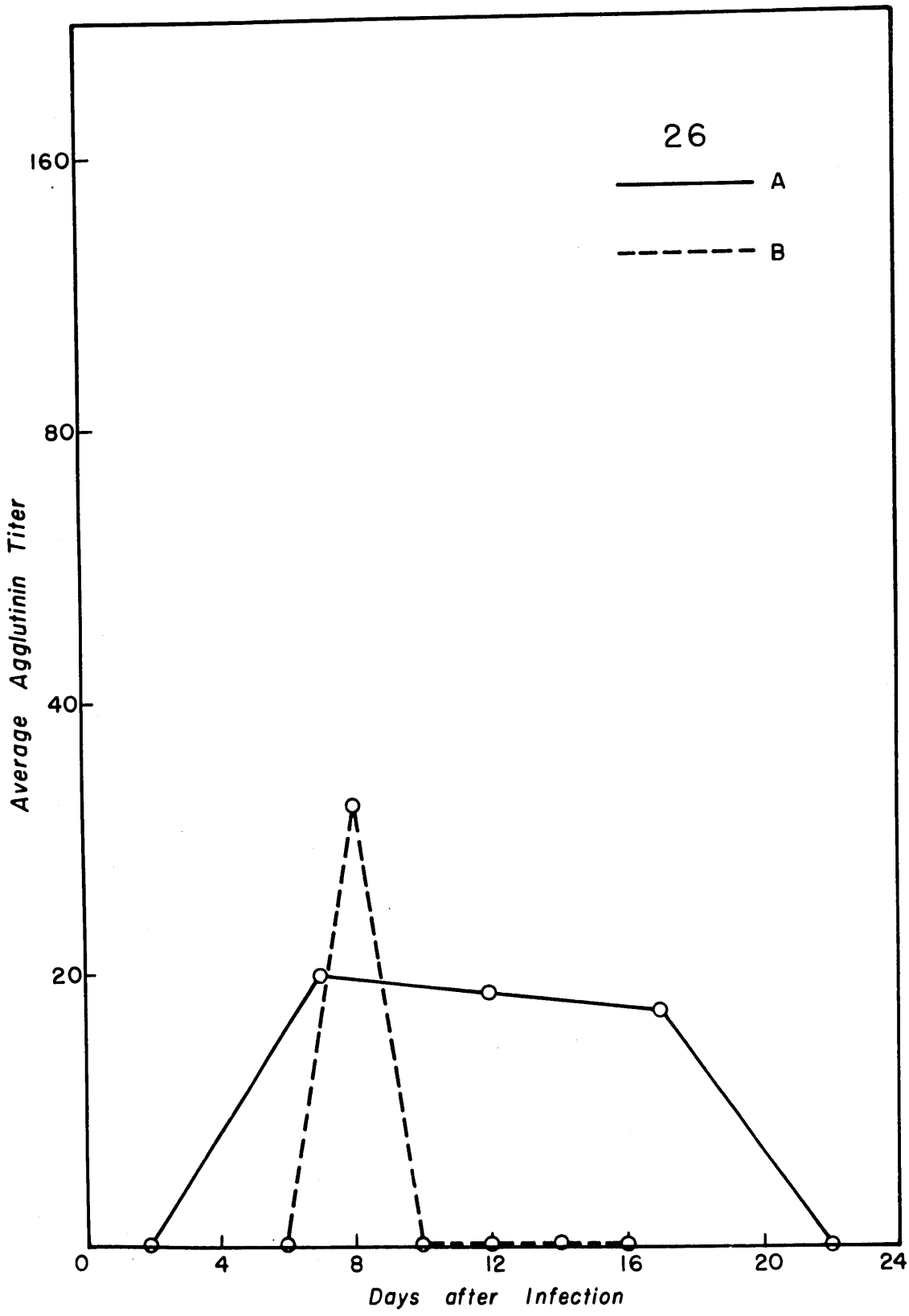
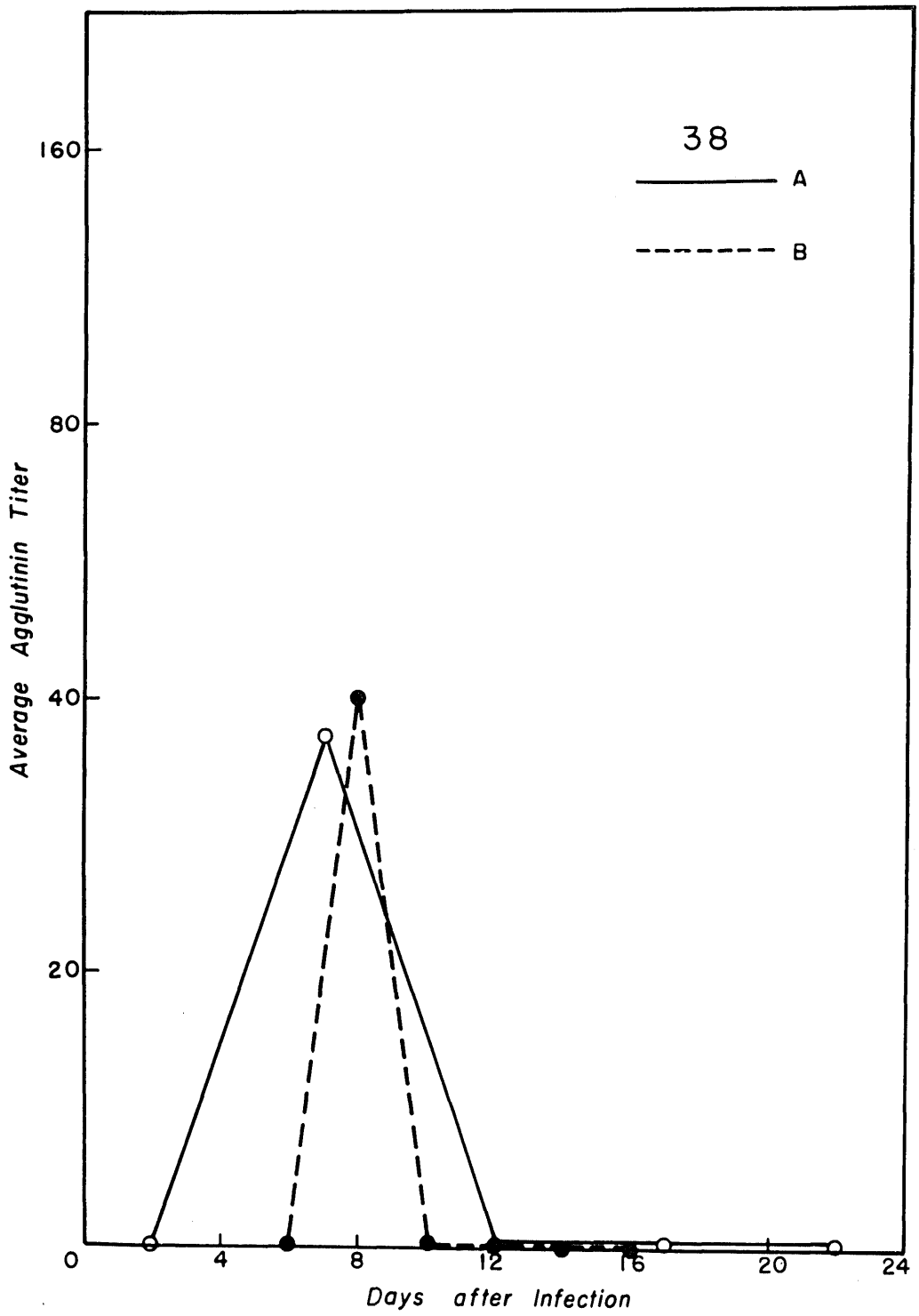

26
A
B
160
80
40
20
0
4
8
12
16
20
24
Average Agglutinin Titer
Days after Infection

Fig. 10 Development of agglutinins in mice following infection with the 38 strain of Bacterium tularense.


38
A
B
160
80
40
20
0
4
8
12
16
20
24
Average Agglutinin Titer
Days after Infection

appeared to be directly related to the degree of immunity elicited in mice by the culture employed.

Streptomycin treatment of white mice following infection with virulent strains of Bacterium tularense

Although living organisms of the various strains of lowered virulence had been shown to elicit good immunologic response in white mice, it was not known whether highly virulent strains possessed this immunogenic property. A major obstacle to the investigation of this problem was the fact that a single fully virulent organism would cause a fatal infection in a mouse. To circumvent this difficulty it was decided to infect a group of white mice with approximately 50 LD_{50} of the Sm strain and follow this with the administration of streptomycin at designated intervals. Originally the mice were infected with approximately 62 Sm organisms and the schedule for streptomycin therapy was arranged as follows:

Dose of streptomycin	Time of administration
10,000 units	48 hrs. after infection
10,000 units	64 hrs. after infection
2,000 units	96 hrs. after infection
2,000 units	168 hrs. after infection
Total 24,000 units	

However, following the administration of the first 20,000 units 17 mice out of 60 under test died non-specific deaths, apparently as a result of streptomycin sensitivity. The remaining mice appeared to be in a highly nervous state and were not in good physical condition at any time during the experiment. Fifteen mice were sacrificed by Mr. Max Moody

for mutation studies on the Sm strain. The remaining mice were divided into two groups of 13 and 15 mice. These were challenged 19 days following the original inoculation with 1,000 and 100 LD_{50} of the Sm strain, respectively. The results are summarized in table 15. It is apparent the mice developed a moderate degree of immunity following infection and streptomycin treatment. The fact that the mice were not in good physical condition before challenge indicates that this may have contributed to the mortality in the group.

A second attempt was made to demonstrate that the Sm strain was highly immunogenic. In this experiment mice were infected with approximately 50 LD_{50} of the Sm strain. In order to minimize the toxic effect of streptomycin for the mice the dosages of streptomycin were reduced and the schedule of treatment was revised as follows:

Dose of streptomycin	Time of administration
5,000 units	48 hours after infection
2,000 units	55 hours after infection
2,000 units	72 hours after infection
2,000 units	80 hours after infection
2,000 units	96 hours after infection
2,000 units	120 hours after infection
2,000 units	144 hours after infection
2,000 units	168 hours after infection
5,000 units	288 hours after infection
total 24,000 units	

In addition to immunity studies quantitative counts of the number of organisms in the mice were determined as previously described, except

Table 15

Resistance of white mice to challenge with virulent *Bacterium tularense* following infection with the Sm strain and streptomycin treatment

Original inoculation	Test dose of virulent organisms (Sm)	
	100 LD_{50}	1,000 LD_{50}
6.2×10^{1}	5/15*	7/13
Normal mice	10/10	

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

that the spleen alone was used as the index of infection. It is known that the organisms persist in this organ longer than in any other part of the body. This procedure simplified the task of following the course of infection without reducing the validity of the results, by eliminating the extra time and materials that would be required if the liver and heart blood were also examined. Three mice were sacrificed and spleen counts were made according to the following schedule:

3 mice - 48 hours after infection and just before streptomycin therapy was begun
3 mice - 1 day after first streptomycin treatment
3 mice - 2 days after first streptomycin treatment
3 mice - 3 days after first streptomycin treatment
3 mice - 4 days after first streptomycin treatment
3 mice - 5 days after first streptomycin treatment
3 mice - 10 days after first streptomycin treatment
3 mice - 11 days after first streptomycin treatment

The last streptomycin treatment was given ten days after therapy was begun. One group of infected mice was not given streptomycin treatment and three of these mice were sacrificed on the 2nd, 3rd, and 4th day following infection. All untreated mice that were not sacrificed died on the fourth day from infection with virulent Bacterium tularense. Nineteen days after infection three mice were sacrificed and quantitative counts were made on the spleen. The remaining mice were challenged at that time with 100 LD_{50} of the Sm strain. Spleen counts were made for three mice on the 1st, 2nd, 3rd, 4th, 5th, 7th, 9th, 11th, 14th, and 16th day following challenge. The remaining mice were saved for survival studies.

On the 16th day after challenge the mice were rechallenged with 100 LD_{50} of the virulent Sm strain. Two mice from this group were sacrificed and quantitative spleen counts were made on the 1st, 2nd, 3rd, 4th, 7th and 10th day following rechallenge. The remaining mice were saved for survival studies.

The results of the quantitative studies are shown graphically in figure 11. The results of the survival studies following challenge and rechallenge are shown in tables 16 and 17.

It may be observed that the streptomycin treatment causes a marked decrease of the organisms in the mice and protects them from death due to infection with Bacterium tularense. Of 97 mice saved for survival studies following streptomycin treatment 87 survived and were used in the subsequent challenge and rechallenge experiments. This represents a survival rate of 89.6 per cent as a result of streptomycin therapy following infection. The streptomycin treatment did not suffice to rid the mice completely of Bacterium tularense. However, the mice appeared to be in good physical condition upon completion of the treatment. It may be seen that the multiplication of the organisms following challenge was significantly lower than that observed following the original infecting dose. This would indicate that the mice had developed considerable immunity to infection.

It may be seen that following rechallenge there was a sharp decrease in the number of organisms followed by a highly irregular fluctuation in the number of organisms present in the mice at any of the given time intervals. The experiment was discontinued 10 days following rechallenge since the remaining animals were to be saved for survival studies.

Fig. 11 Quantitative studies on mice infected with 1.35×10^2 organisms of the Sm strain of Bacterium tularense followed by streptomycin treatment, challenge and rechallenge with the Sm strain. The number of organisms is expressed in curve (a) as the log of the average number found in the spleens of three mice at each interval. Curve (b) represents the log of the number of organisms found in the spleen of one mouse. Curve (c) represents the log of the average number found in the spleens of two mice. Curve (d) respresnts the log of the average number of organisms found in the spleens of mice infected with Sm but not receiving streptomycin.

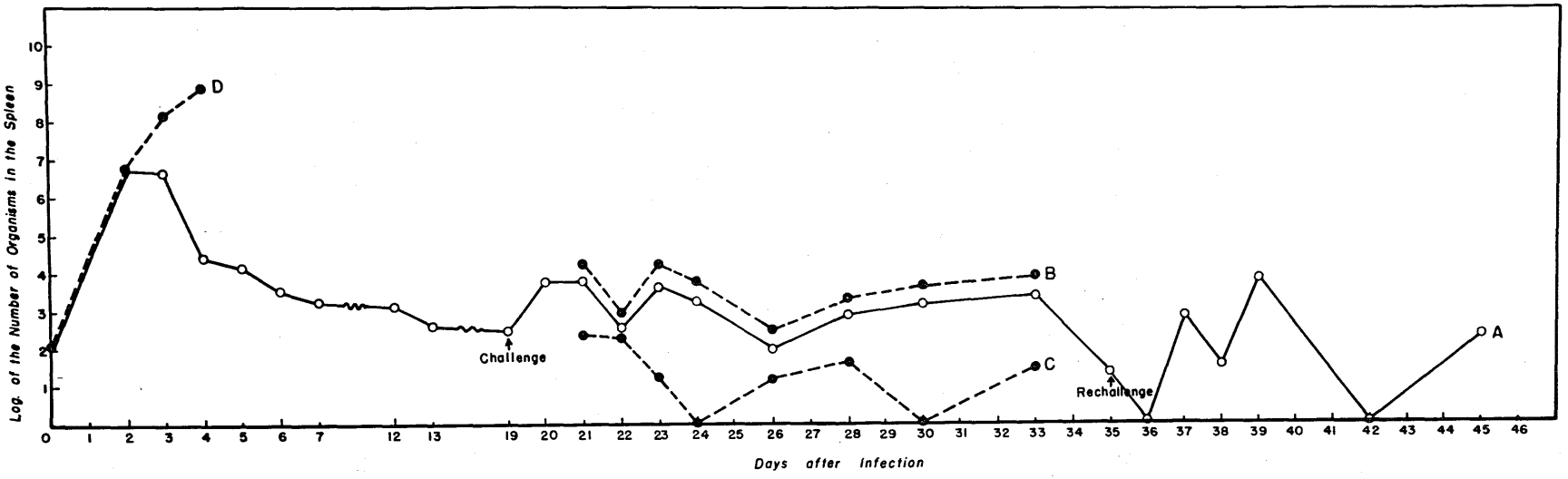

Log. of the Number of Organisms in the Spleen
10
9
8
7
6
5
4
3
2
1
D
B
A
C
Challenge
Rechallenge
0 1 2 3 4 5 6 7 12 13 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46
Days after Infection

Table 16

Resistance of white mice to challenge with virulent Bacterium tularense following infection with the Sm strain and streptomycin treatment

Original Inoculation	Test dose of virulent organisms (Sm) 100 LD_{50}	1,000 LD_{50}
1.35×10^2	14/14*	10/20
	10/10	

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Table 17

Resistance of white mice to rechallenge with virulent Bacterium tularense following original infection with Sm, streptomycin treatment and challenge with Sm organisms

Original Inoculation	Challenge Inoculation	Test dose of virulent organisms (Sm) 100 LD_{50}	1,000 LD_{50}
1.35×10^2	1.4×10^3	—	1/10*
1.35×10^2	1.4×10^2	3/14	—
Normal mice		10/10	

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

It is of interest to note that following challenge only one mouse of each three examined harbored relatively large numbers of organisms, the average being between 1,000 and 9,000 per mouse. The other two mice in each group were shown to have from less than 10 to only 500 organisms, as demonstrated by the quantitative plate counts. The curve shown in figure 11 following challenge, although representing the average of all three mice at each interval, does not represent accurately the individual counts. To show more clearly the actual distribution of infection two accessory curves have been plotted to represent the spleen count for the one highly infected mouse in each group (curve b) and the average count of the two mice which harbored relatively few organisms (curve c). It is evident from curve (b) that the counts obtained from the single mouse in each group served to distort the picture of the actual infection that existed among the three individuals. Curve (c) serves to emphasize the discrepancies in spleen counts that were observed during the period following challenge and prior to rechallenge.

From table 16 it may be seen that the survival rate of these mice is nearly identical with that observed in the previous experiment. This may eliminate the possibility that the toxic effect of the streptomycin played a role in lowering the resistance of the mice to infection with Bacterium tularense in the preceding experiment.

Table 17 shows that a large percentage of the mice were resistant to rechallenge with 100 and 1000 LD_{50} of the Sm strain, indicating that the mice which survived the challenge dose possessed a high degree of immunity to subsequent infection with virulent Bacterium tularense.

The highly virulent Cruse strain of Bacterium tularense was also investigated for its immunogenic properties. Sixty mice were inoculated

with 50 LD_{50} of this strain and the revised schedule of streptomycin treatment was followed, as described above. Six mice died as a result of infection during the course of treatment. Nineteen mice were sacrificed by Mr. Max Moody for mutation studies of the Cruse strain. The remaining 35 mice were divided into two groups of 15 and 20 mice. The mice in each group were challenged with 1000 and 100 LD_{50} of the Sm strain, respectively, 18 days following the original inoculation. Quantitative studies were not done. The results are summarized in table 18. The Cruse organisms appeared to elicit slightly better immunologic response than did the Sm strain. Sixty per cent of the animals survived 1000 LD_{50} and 75 per cent survived 1000 LD_{50} of the challenge strain as compared with 50 and 65 per cent survival in mice immunized with the Sm strain. All normal control mice died as a result of infection with Bacterium tularense.

Two survivors from the group challenged with 1000 LD_{50} were sacrificed 24 days after challenge to determine whether they were still carriers. Spleen counts made as before were negative for Bacterium tularense. Two more mice from this group and two from the group receiving 100 LD_{50} were sacrificed on the 48th day after challenge. The spleen count for one mouse in the former group was over 20,000. Bacterium tularense could not be isolated from the other three mice. These results indicate that individual differences in the mice may determine whether or not the carrier state persists for any length of time.

Twenty-four days after challenge, ten mice of the group receiving 100 LD_{50} were rechallenged with 100 LD_{50} of the Sm strain. Only one mouse died while ten normal control mice infected with the same concentration of organisms all died within 6 days. This is in agreement with the results previously reported for the immunization experiments with the Sm strain.

Table 18

Resistance of white mice to challenge with virulent *Bacterium tularense* following infection with the Cruse strain and streptomycin treatment

Original inoculation	Test dose of virulent organisms (Sm)	
	100 LD_{50}	1,000 LD_{50}
7.1×10^{1}	5/20*	6/15
Normal mice	10/10	

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

A third highly virulent strain, Groves, was also tested for its immunogenic properties. Sixty mice were inoculated with 50 LD_{50} of the Groves strain and treated with streptomycin as previously described. None of the mice died as a result of infection. Mr. Max Moody sacrificed 20 mice for mutation studies on this strain. Nineteen days following infection two groups of 20 mice were challenged with 1000 and 100 LD_{50} of the Sm strain respectively. The results summarized in table 19 show that 85 per cent of the animals survived 1000 LD_{50} while 80 per cent survived 100 LD_{50}. Ten normal control mice challenged with 100 LD_{50} all died.

Fifteen days after challenge two mice from each of the above groups were sacrificed and spleen counts were done as previously described. One mouse of the group receiving 1000 LD_{50} harbored 300 organisms in the spleen, while the second mouse was free from infection. The two mice of the group receiving 100 LD_{50} had spleen counts of over 6000 and 4000, respectively.

Ten survivors from each of the two groups were rechallenged with 1000 and 100 LD_{50} of the virulent Sm strain. All mice in both groups lived, while ten normal control mice infected with 100 LD_{50} died within 6 days. These results indicate that the Groves strain possesses immunogenic properties superior to those of either the Sm or Cruse strain of Bacterium tularense.

Ten days after rechallenge two mice from each group were sacrificed to determine the carrier state. Both mice from the group receiving 1000 LD_{50} harbored over 2000 organisms in the spleens. The mice receiving 100 LD_{50} each harbored approximately 10 organisms.

Table 19

Resistance of white mice to challenge with virulent Bacterium tularense following infection with the Groves strain and streptomycin treatment

Original Inoculation	Test dose of virulent organisms (Sm)	
	100 LD_{50}	1,000 LD_{50}
1.7×10^2	4/20*	3/20
Normal mice	10/10	

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

The persistence of virulent Bacterium tularense in white mice following streptomycin therapy

To determine the carrier state in white mice after streptomycin treatment 20 mice were infected with 7.5×10^1 organisms of the Sm strain. Twenty-four thousand units of streptomycin were administered according to the revised schedule. Three mice were posted on the 16th, 30th, and 35th day following infection. On the 16th day two mice harbored 80 and 70 organisms in the spleen while the third mouse was free from infection with Bacterium tularense. Thirty days after infection one mouse was shown to be harboring 370 organisms in the spleen. Bacterium tularense could not be cultured from the other two mice. On the 35th day the number of organisms found in the three mice was 4340, 340 and 0, respectively.

The passive protection of white mice against virulent Bacterium tularense with immune rat serum

Francis and Felton (35) have reported the use of immune rabbit serum for passive protection of white mice against tularemia. They showed that there was an increase in survival time following infection when immune serum was administered, but actual survival of the mice did not occur. The immune serum they prepared was from rabbits which were immunized with formalized vaccines. It was of interest to determine whether the antibodies induced in rats by living Jap organisms would protect white mice.

Rats weighing approximately 200 grams were inoculated with 4.5×10^7 Jap organisms and 14 days later were bled from the heart. The serum was removed and the samples pooled. The agglutinin titer of the pooled serum for Bacterium tularense was 1:640.

Twenty-four normal mice were inoculated intraabdominally with 0.5 ml of the above serum. The mice were divided into two groups of 12 each which were challenged with 10 and 1 LD_{50} respectively of the Sm strain. Twenty-four normal mice received 0.5 ml of normal rat serum and were challenged as described above. These mice served as the serum controls. For normal controls two groups of 6 mice received 10 and 1 LD_{50} of the Sm strain. The results are summarized in table 20. It may be observed that there was little difference in the mortality between the immune serum mice and the normal serum group; nor was the survival rate significantly superior to that observed in the normal controls. There was very little increase in the survival time of the immunized mice, as compared with either the serum controls or the normal controls. The majority of the immunized mice died 5 days after challenge. Most of the mice in the other groups died 4 days after challenge.

A second experiment was made in order to confirm the above results. The protocol was the same except that 12 additional mice were immunized as previously described and were challenged with 100 LD_{50} of the Sm strain. The results in table 21 confirm the original findings. The survival rate is nearly the same for each group as previously described, and the survival time appeared to average one day longer in the immunized groups than in the control groups.

Table 20

Passive protection of white mice against

Bacterium tularense with Jap-immune rat serum

Original Inoculation	Test dose of virulent organisms (Sm)	
	1 LD_{50}	10 LD_{50}
Jap antiserum	5/12*	12/12
Normal rat serum	10/12	12/12
Normal mice	4/6	6/6

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Table 21

Passive protection of white mice against

Bacterium tularense with Jap-immune rat serum

Original Inoculation	Test dose of virulent organisms (Sm)		
	1 LD_{50}	10 LD_{50}	100 LD_{50}
Jap antiserum	7/12*	11/12	12/12
Normal rat serum		9/12	12/12
Normal mice	5/6	6/6	

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

The use of killed vaccines for the immunization of white mice against Bacterium tularense

Although Coriell et al (7) have reported that killed vaccines prepared from highly virulent strains of Bacterium tularense failed to immunize mice satisfactorily it was decided that killed vaccines of the Jap and Sm strains should be investigated for immunizing potency since living organisms of these strains had been shown to elicit good immunity in mice. Several methods for preparing killed vaccines with both strains were employed. The designation of the vaccines studied and the method for the preparation of each is given below.

Acetone Extracted Vaccine No. 1. (A. E. V. #1)

Jap cultures were grown for 48 hours in Snyder's broth (41) and 25 per cent by volume of C.P. acetone was added and allowed to stand overnight at 4°C. This was centrifuged and washed twice in 10 to 15 ml of saline. After the second washing the cells were resuspended in 0.1 per cent formalized saline and placed in the refrigerator until used.

Acetone Extracted Vaccine No. 2. (A. E. V. #2)

This vaccine was prepared according to the method used in A. E. V. #1 except that the Jap strain was grown in Mills' medium (46) for 24 hours.

Acetone Extracted Vaccine No. 3. (A. E. V. #3)

A 24 hour culture of the Jap strain was grown in Mills' medium and the cells were centrifuged, washed once in saline, suspended in 20 ml of 90 per cent acetone and allowed to stand overnight at room temperature. The cells were again centrifuged and the acetone decanted. The cells were then washed three times in saline, and resuspended in 0.1 per cent formalized saline.

Acetone Extracted Vaccine No. 4. (A. E. V. #4)

A 24 hour culture of the Sm strain grown in Mills' medium was treated as described for the preparation of A. E. V. #3.

Acetone Extracted Vaccine No. 5. (A. E. V. #5)

A 24 hour culture of the Sm strain grown in Mills' medium was treated as described for the preparation of A. E. V. #3 and #4 except that 75 per cent acetone was used for extraction. All of these vaccines were found to be sterile when plated directly on GCBA. In the experiments in which these vaccines were employed the mice were immunized with injection of 0.5 ml of a standard suspension of the vaccines on each of three alternate days. The mice were challenged with from 10 to 10,000 LD_{50} of the Sm strain. The results of all of these experiments are summarized in table 22.

Sonically Extracted Vaccines

Since the use of high frequency sound waves has been employed successfully in extracting the labile VI antigen from cells of Salmonella typhi it was decided to determine whether this means of extraction could be used for the preparation of potent Bacterium tularense vaccines.

Sonic Extract No. 1. (S. E. #1)

The cells of 24 hour Jap cultures grown in Snyder's broth were harvested by centrifugation and were resuspended in sterile saline to a concentration of 2.0×10^{10} organisms per ml. This suspension was subjected to sonic vibration for 50 minutes at 9000 cycles in a Raytheon Sonic Oscillator. Following sonic disintegration the suspension was filtered through a Seitz filter and frozen. Plate counts showed the suspension to be completely sterile at time of use.

Twenty mice were inoculated with 0.5 ml of the undiluted suspension.

Table 22

Resistance of white mice to challenge with virulent Bacterium tularense following vaccination with acetone extracted vaccines

Vaccine	Days after inoculation	Test dose of virulent organisms (Sm) 10 LD_{50}	100 LD_{50}	1,000 LD_{50}	10,000 LD_{50}
#1 (Jap)	22	7/10*	10/10	10/10	3/3
#2 (Jap) (a)	20	2/10	7/10	—	—
(b)	16	—	7/10	—	—
#3 (Jap)	15	—	18/19	—	—
#4 (Sm)	15	—	10/10	—	—
#5 (Sm)	17	—	18/19	—	—
Normal mice		17/18	15/15	—	—

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Twenty-one days later the mice were divided into two groups of 10 mice each which were challenged with 100 and 10 LD_{50} of the Sm strain, respectively. The results are summarized in table 23.

Sonic Extract No. 2. (S. E. #2)

This vaccine was prepared in the same manner as S. E. #1 except that the original suspension contained 6.5×10^{10} organisms per ml.

A group of 10 mice received 0.5 ml of the undiluted extract on each of three alternate days. Eighteen days after the first inoculation the mice were challenged with 100 LD_{50} of the Sm strain. These results are also summarized in table 23.

Sonic Extract No. 3. (S. E. #3)

This vaccine was prepared as previously described except that the original suspension contained 12.0×10^{10} organisms per ml.

Ten mice were inoculated with 0.5 ml of the undiluted extract on each of three alternate days. Sixteen days after the first inoculation the mice were challenged with 100 LD_{50} of the Sm strain. The results may be found in table 23.

Sonic Extract No. 4. (S. E. #4)

This vaccine was prepared as previously described except that the cells were grown in Mills' medium and the original suspension contained 2.4×10^{10} Jap organisms. Living organisms of the avirulent 38 strain were then added to give a concentration of 2.9×10^{9} cells per ml and allowed to stand at room temperature for one hour. Following the absorption procedure 25 mice were each inoculated with 0.5 ml of the suspension. Ten days later the mice were challenged with 100 LD_{50} of the virulent Sm strain. These results are included in table 23.

Table 23

Resistance of white mice to challenge with virulent Bacterium tularense following vaccination with sonic extracts

Vaccine	Days after inoculation	Test dose of virulent organisms (Sm) 10 LD_{50}	100 LD_{50}
S. E. #1	21	4/10*	6/10
S. E. #2	18	—	8/10
S. E. #3	16	—	9/10
S. E. #4 + Living "38"	10	—	18/20
Normal mice		22/24	—

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

Sodium Fluoride Killed Vaccine

Sodium fluoride is known to inhibit the action of the cytochromes without denaturing cellular protein. It was thought that by the use of this cell poison it might be possible to prepare a killed vaccine that had not lost its antigenic properties. Accordingly a 24 hour Jap culture grown in Mills' medium was centrifuged and the cells resuspended in one per cent sodium fluoride for 24 hours. The cells were then centrifuged and washed 4 times with physiological saline. They were then resuspended in 0.5 per cent formalized saline. Plate counts showed that the vaccine was sterile. Nineteen mice each received 0.5 ml of a standard suspension of the vaccine on each of 3 alternate days. The mice were challenged 22 days after the first inoculation with 100 LD_{50} of the Sm strain. The results are summarized in table 24.

Streptomycin Killed Vaccine

It has been shown that streptomycin exerts a bactericidal effect on Bacterium tularense. The exact mechanism of its action is not known, but it seems probable that the cellular protein is not denatured by contact with streptomycin. Assuming this to be true it seemed possible that potent killed vaccines might be prepared. A 24 hour Jap culture grown in Mills' medium was centrifuged and the cells were resuspended to a concentration of approximately 3.0×10^9 organisms per ml in saline containing 20,000 units of streptomycin per ml. The suspension was allowed to incubate at room temperature overnight. The cells were centrifuged and washed three times and resuspended in 0.1 per cent formalized saline. The vaccine was found to be sterile as determined by plate count.

Nine mice were each inoculated with 0.5 ml of the standard suspension of the organisms on each of 3 alternate days. Twenty-two days

Table 24

Resistance of white mice to challenge with virulent Bacterium tularense following vaccination with sodium fluoride and streptomycin killed vaccines

Vaccine	Days after inoculation	Test dose of virulent organisms (Sm) 100 LD_{50}
Na F	22	16/19*
Streptomycin	22	9/9
Normal mice		5/5

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

after the first inoculation they received 100 LD_{50} of the virulent Sm strain. These results are shown in table 24.

Tissue Extract Antigens

The predominantly negative results obtained in trying to prepare a potent killed culture vaccine against Bacterium tularense suggested the possibility that the organisms do not produce the immunizing factor when grown in the usual culture media, but that they may produce this antigen in vivo.

Eight guinea pigs were inoculated with 4.4×10^5 Jap organisms and three days later were sacrificed. The peritoneal cavity of each pig was washed with 10 ml of saline. The washings were combined and filtered through a Seitz filter. The number of organisms prior to filtration was found to be approximately 340,000 per ml. Ten mice were inoculated with 0.5 ml of the sterile undiluted filtrate on each of 3 alternate days. Seventeen days after the first inoculation the mice received 100 LD_{50} of the Sm strain. The results are shown in table 25.

The spleens of the 8 guinea pigs were removed and immediately frozen over dry ice. They were then macerated with chilled mortar and pestle and suspended in ice cold saline. The brei was centrifuged and the supernatant filtered through a chilled Seitz filter. The filtered material was frozen in the CO_2 box until used. Plate counts made prior to use showed the filtrate to be sterile. Ten mice were inoculated with 0.5 ml of the spenic extract on each of 4 alternate days. Twenty days after the first inoculation the mice were challenged with 100 LD_{50} of the Sm strain of Bacterium tularense. These results may be found in table 25.

From an examination of tables 22, 23, 24 and 25 it may be seen that none of the methods for preparing killed vaccines gave satisfactory

Table 25

Resistance of white mice to challenge with virulent Bacterium tularense following vaccination with tissue extracts of guinea pigs infected with the Jap strain

Vaccine	Days after inoculation	Test dose of virulent Organisms (Sm) 100 LD_{50}
I.P. Washings	17	10/10*
Splenic extracts	20	10/10
Normal mice		20/20

* Mortality ratio = $\frac{\text{Dead}}{\text{Tested}}$

results. Mice immunized with these vaccines were resistant to only very low concentrations of the Sm organisms or showed no resistance other than a small increase in survival time following infection. The survival rates of these mice were discouragingly low as compared to the survival rates obtained using living Jap or Russ organisms.

The longest survival time was observed in the mice immunized with the sodium fluoride killed cells. The majority of these mice did not die until the eighth or ninth day following challenge. It should also be mentioned that these mice did not show any of the usual signs of illness until just before death. These observations indicate that this method for preparing vaccines would bear further study. However, neither of the vaccines prepared using methods designed to prevent the denaturation of cellular protein were satisfactory.

The results obtained using tissue extracts were completely negative. Nevertheless, further work should be done before eliminating tissue extracts of infected animals as a possible source of potent Bacterium tularense antigen.

DISCUSSION OF RESULTS

The results of this investigation have shown that certain marked differences exist among the strains of Bacterium tularense studied. They disclose the fact that living organisms of some of the strains elicit good immunogenic response in white mice while others do not do so. An inspection of tables 2, 3, 5 and 7 reveals that the immunogenic properties for white mice of the Jap and Russ strains are far superior to those of Ohara, 26, Max and 38. It is also of importance to note that the LD_{50} of these latter strains is less than that of the Jap and Russ strains.

The completely avirulent strain 38 exhibits the least immunogenic potency. Ohara, 26, and Max are endowed with slightly greater immunizing capacity, while the Russ and Jap strains both appear to be highly immunogenic. The observation that living cells of some strains of lowered virulence elicit good immunologic response in mice somewhat parallels the findings of Jawetz and Meyer (20) who demonstrated that living avirulent organisms of Pasteruella pestis would immunize guinea pigs against bubonic plague while killed vaccines would not do so. The unsuccessful use of killed vaccines for immunizing white mice against Bacterium tularense will be discussed later.

From tables 11, 13 and 14 it may be observed that differences also exist among the highly immunogenic strains. Initial doses consisting of large concentrations of the Russ and Depue organisms are necessary to elicit good immunological response in mice while relatively few Jap and RI cells are required to protect these animals against infection with the virulent Sm strain. The Carr strain, which had been shown by other workers in this laboratory to be highly immunogenic when large numbers of organisms were inoculated into mice, did not provide

good protection when only 2.0×10^4 organisms were injected.

A comparison of figures 1, 3, 4 5 and 6 with tables 5, 13 and 14 serves to emphasize the close relationship between the ability of a strain to multiply in the animal body and its immunogenic power. The Jap and RI strains multiplied freely in mice at all concentrations while the Russ strain multiplied to a much lesser degree or not at all depending upon the original concentration of cells injected. The Depue strain appeared to lack completely any ability to multiply in mice. The number of organisms in the spleen was always found to be less than the infecting concentration when this strain was studied. This strain is slightly more virulent than strain 38 and is shown to elicit a greater immunologic response in white mice.

The three tables show that the Jap and RI strains elicit good immunity in mice even when low concentrations of organisms are used for inoculation. The Russ and Depue strains elicit good immunologic response if extremely high concentrations of organisms are used for vaccination. When small numbers of these organisms are used little or no multiplication occurs and little immunity is produced. However, Russ was shown to be slightly more virulent and to possess somewhat greater immunogenic power than the Depue strain. Strain 38 elicits the least immunogenic response of the five strains even when a series of inoculations with large numbers of this organism is employed. This strain is totally unable to establish itself in mice and has been shown to disappear from the animal body within 6 days following the inoculation of 1.5×10^9 organisms.

These results emphasize that a direct relationship exists between virulence and the ability of the strains to multiply in mice, with the immunogenic properties of these strains. They also present indirect

evidence that the differences in antigenicity are of a quantitative nature and that qualitative antigenic differences between the strains have not been demonstrated.

Other important data have been obtained from studies of the Jap culture. The original discovery of the immunogenic power of living organisms was made with this strain and for this reason it was selected for extensive study.

The immunity elicited following inoculation with the Jap strain appears to last for at least 4 months. As shown in table 8 the mice seemed to be well protected during and at the expiration of this period. There is nothing to suggest that this immunity ever disappears completely, although further studies were not made. Buchele and Downs (23) have shown that rats exhibit resistance to infection with virulent Bacterium tularense up to 9 months after immunization. The immunity elicited by the Jap strain appears to be quite solid since Jap immunized mice are highly resistant to rechallenge with the virulent Sm strain as may be observed in table 6. Similar results have been obtained with rats by Buchele and Downs (23). It was not possible to measure the cumulative effect of the challenge and rechallenge doses on the degree of immunity in the vaccinated mice.

Mice immunized by living organisms of the Jap strain are resistant to infection with more than one virulent strain as shown by the results summarized in table 4. The mice were able to resist infection with tremendous concentrations of the Groves, Cruse and Ince strains. These findings are in accord with the conclusions drawn by Foshay (4). It is his contention that upon recovery from tularemia, humans are immune to subsequent infection with any strain of Bacterium tularense.

These results strongly suggest that there is very little qualitative antigenic difference among the various strains.

It is of interest to note that Coriell et al (7) could not demonstrate solid immunity to tularemia in mice which were immunized with killed vaccines. They observed that the few survivors of a challenge dose of virulent Bacterium tularense remained susceptible to tularemia and usually succumbed when rechallenged.

The appearance of immunity in white mice is extremely rapid and it was shown that an excellent degree of immunity could be demonstrated as early as 4 days after vaccination with the Jap strain. Some evidence of immunologic response was observed as early as the 2nd and 3rd day after inoculation (table 9). It is of interest to note that the multiplication of Jap organisms usually reached a peak between the 3rd and 5th day after infection. In contrast to this, it was not possible to demonstrate agglutinins in vaccinated mice earlier than the 7th or 8th day after inoculation. As few as 20 living Jap organisms provided mice with a fair degree of immunity and any number of cells between 200 and 200,000 insured a high degree of protection against infection with the Sm strain. As has been previously mentioned, the Jap strain multiplies freely in mice. Other immunogenic strains have not elicited such good immunologic response when small numbers of organisms were inoculated into mice.

Additional evidence for the solid immunity provided by vaccination with the Jap strain is presented in table 12 and figure 2. These results indicate that immunized mice are able to rid themselves rapidly and efficiently of virulent organisms following infection. Saline suspensions prepared with the spleens of immunized, and subsequently challenged,

mice were generally non-infective for normal mice as early as 4 days following challenge. Since a single virulent organism can cause a fatal infection in normal mice, this supports the conclusion that the Jap strain provided these animals with a solid immunity to infection with Bacterium tularense. In contrast to normal mice, Jap immunized animals were able to limit the multiplication of virulent organisms and eventually rid themselves of these organisms, as shown by bacterial counts of the heart blood, spleen and liver.

Foshay (24) has suggested that immunity to tularemia is dependent upon the continuous presence of living organisms in the body. The carrier state in rats and mice has been determined frequently and it is known that highly virulent organisms may persist in the recovered animals for long periods of time. On the other hand, it has not been possible to demonstrate the presence of living Jap organisms in mice longer than 18 days after inoculation. Since Jap immunized animals are resistant to fatal infection with virulent Bacterium tularense for several months this may indicate that immunity is independent of the carrier state. However, the possibility exists that the plating technic used to determine carriers is not sufficiently delicate to detect very small numbers of living Jap cells. If this is true one should not eliminate the carrier state as a possible mechanism by which immunity to tularemia is maintained.

It has been possible to correlate the amount of agglutinin production in mice following the inoculation of strains of lowered virulence with their virulence and immunogenic properties. Strains Jap, Russ, 26, and 38 were selected for agglutinin studies and it may be observed in figures 7, 8, 9 and 10 that only the highly immunogenic Jap and Russ cultures elicited an appreciable agglutinin response. Strains

26 and 38 failed to do so. In all cases, however, the agglutinin titers remained low in contrast to the relatively high titers that may be obtained in rats and other laboratory animals. These results indicate that the agglutinin titer of mouse serum is not a satisfactory index of immunity against tularemia.

It was considered necessary to determine the immunogenic capacity of the highly virulent strains that were available. Streptomycin treatment following infection with the Sm, Cruse and Groves strains showed that the infection could be controlled after an initial period of cell multiplication in the mice. Challenge of these mice with the Sm strain demonstrated that they had developed some degree of immunity following infection during the course of streptomycin therapy. Upon rechallenge the mice appeared to be even more resistant to fatal infection. Once again the cumulative effect of the challenge doses on the degree of immunity could not be evaluated.

Streptomycin was shown to aid in ridding the mouse of infection (figure 11). The initial effect appeared to be bacteriostatic in character, and a 24 hour period during which the organisms in the animals remained constant, neither increasing nor decreasing in number, was observed. This was followed by a rapid and regular decrease for 5 days following the initiation of streptomycin treatment after which the number of residual organisms leveled off and remained relatively constant until the animals were challenged 19 days after infection. It is important to note that the mice were able to limit multiplication of the organisms following challenge, the average number of organisms never reaching a concentration equal to the log. 4. This is also true upon rechallenge of the survivors. Noticeable fluctuation in the number of organisms found in the individual mice were observed

but they all fell within a low and predictable range.

The two accessory curves (b) and (c) shown in figure 11 were plotted to demonstrate that a single mouse harboring from 3,000 to 10,000 organisms may mask, or distort, the results obtained from the other individuals examined at any given time. The curve plotted for the average number of organisms in 3 mice at each stated interval does not differ greatly from curve (b), yet it is obvious that marked discrepancies exist between curves (A) and (B) on the one hand, and curve (c) on the other. These results are mentioned in order to bring out the fact that, generally, one mouse of every three challenged with 100 LD_{50} of the Sm strain harbored relatively large numbers of virulent Bacterium tularense organisms. This ratio also held true for the number of deaths and survivals following challenge with 100 LD_{50}, as may be seen in tables 15 and 16. A slightly better survival rate following challenge was observed when the Cruse strain was tested for its immunogenic properties. However, the Groves strain elicited the best immunogenic response of all the highly virulent strains. A comparison of tables 15 through 19 will show that some immunogenic differences may exist even among highly virulent strains whose virulence is not significantly different.

Upon rechallenge all mice immunized with the 3 strains were highly resistant to fatal infection. The survival rate in each case was from 80 to 100 per cent. It must be emphasized again that the cumulative effect of these successive infecting doses on the immunity of the animals cannot be measured.

It is not possible to evaluate the effect that streptomycin therapy might have had in depressing the immunologic response of the mice. Presumably, the various groups of mice employed may have reacted differently

to the known toxic effects of the streptomycin, or they may have responded differently to the immunogenic stimuli in the presence of this antibiotic. All of these factors should be considered before concluding that these strains are not as highly immunogenic as the Jap strain, or that they differ among themselves in this respect.

The streptomycin schedule that was ultimately adopted as standard procedure appeared to be quite satisfactory for the protection of the mice from fatal infections since a survival rate of approximately 90 per cent was obtained consistently. Too great a concentration of streptomycin evokes neuro-toxic symptoms, and often death, in the mice. Also, higher concentrations of this drug may reduce the infection too rapidly in which case the organisms might not multiply enough to elicit good immunity in the animals.

Following streptomycin treatment the mice were shown to harbor the virulent organisms up to 30 days. Apparently, in the carrier state, a satisfactory balance had been reached and was maintained indefinitely between the mice and the infecting agent.

The results of the experiments on the passive protection in mice shown in tables 20 and 21 indicate that relatively high agglutinin titers of immune serum are not an index of potent protective capacity of the serum. Protective antibodies were not apparent in the rat serum employed. It should be mentioned that this serum was obtained from rats recovered from infection with the Jap strain of Bacterium tularense and did not constitute a hyper-immune serum. A slight delay in the death of the mice treated with the immune rat serum was observed, averaging approximately one day over that of the control groups. The death rate was approximately the same in the test and control groups. These results are in accord

with those of Francis and Felton (35) who demonstrated a short delay following infection in the time of death of mice treated with immune serum. The death rate was identical with that of the control animals.

Experiments with killed culture vaccines using strains of low virulence, and fully virulent strains indicate that such vaccines produce little immunity in the white mouse. These vaccines, however, are effective in the white rat as shown by Downs et al (6). They are also partially successful immunizing agents in man as indicated by the reports of Foshay (4), and Kadul et al (5). However, immunization of rats and man with killed cultures does not appear to confer as solid an immunity as that following infection. Infection with the living organisms appears to be necessary to the production of immunity in the mouse. This may indicate, as Jawetz and Meyer (20) have proposed with regard to Pasteurella pestis, that there is a very labile immunogenic constituent present in the living culture which is denatured wholly or in part by methods used for killing the organisms. On the other hand, there may be an effective immunogenic substance produced in the animal which is not present in artificial culture. In more resistant animals as in man and rats, killed vaccines may be effective because these animals respond to small amounts of the denatured antigen or because the killed vaccines contain antigenic constituents to which the mouse does not react.

The vaccines prepared by the various methods of acetone extraction using both the Jap and Sm strains did not elicit good immunity in mice (table 23). The preparation of vaccines with sonically treated organisms of the Jap strain was prompted by the work of Chambers and Florsdorf (32) who reported that they could successfully extract the highly labile Vi antigen of Salmonella typhosa. Unfortunately such a technic does not

appear practicable for Bacterium tularense. The vaccines which were prepared in this manner failed to protect mice satisfactorily as may be observed in table 23.

The use of enzyme inhibitors that do not denature cell protein was investigated since it was thought that possibly the methods of acetone extraction and sonic disintegration might disrupt and damage the antigenic constituent of the organisms. Streptomycin was used as the killing agent in the preparation of one of these vaccines and sodium fluoride in another. It was not possible to prepare a potent vaccine for immunizing mice using these technics. Tables 24 and 25 record the unsuccessful attempts with these vaccines. The mice that were immunized with the sodium fluoride-killed cells showed a significantly prolonged survival time and never appeared ill until just prior to death. It would seem that the preparation and use of this vaccine should be investigated at greater length.

Since none of the killed vaccines prepared by these various methods were potent immunizing agents for mice, the possibility existed that the immunizing antigen of Bacterium tularense was not produced by the organisms when grown in the usual culture media but might be produced in the tissues of the animals they infect. Some basis for this view may be derived from the work of Cromartie et al (27) who reported the production of an immunizing substance by Bacillus anthracis in animal tissue which was not observed when the organism was grown in ordinary culture media.

The use of splenic extracts and filtered peritoneal washings from guinea pigs infected with the Jap strain of Bacterium tularense for immunizing mice was not successful. There may be several factors responsible for this. First, the number of organisms in the extracts did not reach the concentration desired; secondly, the antigen may have been removed in

large part when the spleen suspensions and peritoneal washings were filtered through Seitz filters; thirdly, the antigen could conceivably be destroyed by the action of body enzymes, proteinases or carbohydrases, present in the extracts before they were used for vaccination. The preparation of tissue extracts should be investigated more thoroughly before discarding this method as unsatisfactory.

In general, the results of this investigation have emphasized the lack of any qualitative antigenic difference among the various strains of Bacterium tularense. The differences that do exist are probably quantitative. The results of the infectivity experiments, and the immunization of white mice with living and killed organisms bear out such a conclusion. It is quite apparent that the virulence, and consequently the ability of the strains to multiply in mice, is directly responsible for the immunogenic quality of any given strain. All gradations of virulence and immunogenic power are represented by the strains studied in this investigation.

Because of its high susceptibility to tularemia the white mouse was chosen as the test animal in this study. The results have suggested that it is a delicate indicator for determining antigenic differences among various strains of Bacterium tularense. Some laboratory animals are relatively resistant to, and easily immunized against, tularemia as in the case of the white rat. Others such as the rabbit and guinea pig are highly susceptible and are not protected by either living or killed Bacterium tularense vaccines.

Although the white mouse does not respond immunologically to killed Bacterium tularense it appears to be highly responsive to antigenic differences of living organisms. Accordingly, the white mouse appears to be the most satisfactory laboratory animal for this study.

SUMMARY

1. Living organisms of certain strains of Bacterium tularense were found to elicit a high degree of immunity in white mice when inoculated in sub-lethal concentrations.

2. It was possible to detect immunogenic differences for white mice among the various strains tested.

3. The immunogenic differences could be correlated directly with the virulence of the strains and their ability to multiply in mice.

4. The vaccination of white mice with living organisms of the Jap strain produced a high degree of immunity within three days following inoculation.

5. As few as 20 living cells of the slightly virulent strain, Jap, elicited good immunity when inoculated into mice. Moreover, this strain of Bacterium tularense provided white mice with an immunity against more than one highly virulent strain.

6. Mice immunized with living organisms of the Jap strain were found to possess a high degree of immunity 4 months after vaccination.

7. Living organisms of the Jap strain could not be recovered from the spleens of mice longer than 18 days after inoculation.

8. White mice immunized with living Jap organisms were shown, upon challenge, to rid themselves quickly and efficiently of highly virulent Bacterium tularense.

9. Strains of high and moderate virulence were shown to multiply freely in normal mice while strains of very low virulence showed a greatly reduced ability to establish themselves in mice.

10. It was possible to show a correlation between the production of agglutinins in mice and the immunogenic properties of strains Jap,

Russ, 26 and 38 for these animals.

11. It was not possible to protect mice passively against highly virulent Bacterium tularense with the serum of rats recovered from Jap infections.

12. Organisms of the highly virulent strains could be recovered from immunized mice up to 24 days after challenge.

13. Killed culture vaccines did not elicit good immunologic response in white mice.

14. The antigen responsible for the production of immunity in mice was either destroyed during the preparation of the killed vaccines or it was not produced in vitro.

15. It was not possible to isolate an immunizing antigen from the tissues of guinea pigs infected with the Jap strain of Bacterium tularense.

16. By protecting mice with streptomycin from fatal infection it was possible to demonstrate that highly virulent strains of Bacterium tularense elicit good immunity in white mice.

17. The immunity elicited in mice by living organisms of the immunogenic strains appears to be quite solid as evidenced by a high survival rate following rechallenge of the immunized animals.

18. White mice were shown to be highly satisfactory test animals for the purposes of this study.

REFERENCES

1. Francis, E., and Evans, A. 1926 Agglutination, cross agglutination and agglutination absorption in tularemia. Public Health Reports, 41, 1273-1295.

2. Foshay, Lee Unpublished material.

3. Downs, C. M. Unpublished material.

4. Foshay, Lee Unpublished material.

5. Kadull, P. J., Reams, H. R., Coriell, L. L., and Foshay, Lee Studies on tularemia, V: Immunization of man. (To be published).

6. Downs, C. M., Coriell, L. L., Eigelsbach, T. H., Plitt, K. F, Pinchot, G. B., and Owen, B. J. 1947 Studies on tularemia, I: Immunization of white rats. Jour. Immunol., 56, 229-243.

7. Coriell, L. L., Downs, C. M., and Clapp, M. P. 1947 Studies on tularemia, II: Immunization of mice. Jour. Immunol., 56, 245-253.

8. Chapman, S. S., Downs, C. M., Coriell, L. L., and Kowal, S. S. Streptomycin studies in tularemia. (To be published).

9. Bond, G. C., and Downs, C. M. 1935 Antigenic studies on the genus Pasteurella. Trans. Kans. Acad. Sci., 38, 87-92.

10. Francis, E. 1941 Fermentation of sugars by Bacterium tularense. Jour. Bact., 43, 343-346.

11. Downs, C. M., and Bond, G. C. 1935 Studies on the cultural characteristics of Pasteurella tularense. Jour. Bact., 30, 485-490.

12. Green, R. G. 1943 Virulence of tularemia as related to animal and arthopod hosts. Am. Jour. Hyg., 38, 282-292.

13. Davis, G. E., Philips, C. B., and Parker, R. R. 1934 The isolation from the Rocky Mountain wood tick (Dermacentor andersoni) of strains of Bacterium tularense of low virulence for guinea pigs and domestic rabbits. Am. Jour. Hyg., 19, 449-456.

14. Foshay, Lee 1932 Induction of avirulence in Pasteurella tularensis. Jour. Inf. Dis., 51, 280-285.

15. Jellison, W. L., and Parker, R. R. 1945 Rodents, rabbits and tularemia in North America: Some zoological and epidemiological consideration. Am. Jour. Trop. Med., 25, 349-362.

16. Lillie, R. D., and Francis, E. 1936 The pathology of tularemia. Nat. Inst. Health Bul. 167.

17. Downs, C. M. 1932 Immunological studies on tularemia in rabbits. Jour. Inf. Dis., 51, 315-323.

18. Downs, C. M., Coriell, L. L., Pinchot, G. B., Maumenee, E., Klauber, A., Chapman, S. S., and Owen, B. 1947 Studies on tularemia, I: The comparative susceptibility of various laboratory animals. Jour. Immunol., 56, 217-228.

19. Larson, C. L. 1945 Immunization of white rats against infections with Pasteurella tularensis. Pub. Health Reports, 60, 725-734.

20. Jawetz, E., and Meyer, K. 1943 Avirulent strains of Pasteurella pestis. Jour. Inf. Dis., 73, 124-143.

21. Downs, C. M., Buchele, L., and Edgar, E. P. 1949 Studies on pathogenesis and immunity in tularemia, I: The pathogenesis of tularemia in the white rat. In press.

22. Jawetz, E., and Meyer, K. 1944 The behavior of virulent and avirulent Pasteurella pestis in normal and immune experimental animals. Jour. Inf. Dis., 74, 1-13.

23. Buchele, L., and Downs, C. M. 1949 Studies on pathogenesis and immunity in tularemia, II: Immune response of the white rateto Bacterium tularense. In press.

24. Foshay, Lee 1936 Viability of Bacterium tularense in human tissues. Jour. Am. Med. Assoc., 106, 2141-2143.

25. Downs, C. M. 1949 Unpublished material.

26. Foshay, L., Hesselbrock, W. H., Wittenberg, H. J., and Rodenberg, H. A. 1942 Vaccine prophylaxis against tularemia in man. Am. Jour. Pub. Health, 32, 1131-1145.

27. Elbert, B. Y. (Frunze) 1945 The experimental skin method of vaccination against tularemia. Jour. Microbiology, Epidemiology, Immunobiology. Nat. Committee of Health Protection U. S. S. R., 12, 87-89.

28. Elbert, B. Y., and Gaiskii, N. A. (Irkutck) 1945 Mechanism of infection and immunity in experimental tularemia. Jour. Microbiology, Epidemiology, Immunobiology. Nat. Committee of Health Protection U. S. S. R., 7-8, 55-56.

29. Cromartie, W. J., Watson, D. W., Bloom, W. L., and Heckley, R. J. 1947 Studies on infection with Bacillus anthracis II The immunological and tissue damaging properties of extracts prepared from lesions of Bacillus anthracis infection. Jour. Inf. Dis., 80, 14-27.

30. Gladstone, G. P. 1946 Immunity to anthrax: Protective antigen Brit. Jour. Exp. Path., 27, 394-418.

31. Felix, A. 1934 A new antigen of Bacillus typhosus. Lancet, 227, 186-191.

32. Chambers, L. A., and Florsdorf, E. W. 1936 Sonic extraction of labile bacterial constituents. Proc. Soc. Exp. Biol. and Med., 34, 631-636.

33. Foshay, Lee 1932 Serum treatment of tularemia. Jour. Am. Med. Assoc., 98, 552-553.

34. Foshay, Lee 1934 Tularemia treated by a new specific antiserum. Am. Jour. Med. Sci., 187, 235-245.

35. Francis, E., and Felton, L. D. 1942 Antitularemic serum. Pub. Health Reports, 57, 44-45.

36. Foshay, L., and Pasternack, A. B. 1946 Streptomycin treatment of tularemia. Jour. Am. Med. Assoc., 130, 393-398.

37. Foshay, Lee 1946 A comparative study of the treatment of tularemia with immune serum, hyperimmune serum and streptomycin. Am. Jour. Med., 1, 180-188.

38. Foshay, Lee 1947 Treatment of tularemia with streptomycin. Am. Jour. Med., 2, 467-473.

39. Heilman, F. R. 1944 Streptomycin in the treatment of experimental tularemia. Proc. Staff Meetings Mayo Clinic, 19, 553-559.

40. Chapman, S. S., Downs, C. M., Coriell, L. L., and Kowal, S. S. 1946 Streptomycin studies in tularemia. I The effect of streptothricin and streptomycin on Bacterium tularense in vitro. and in vivo (mouse). (To be published)

41. Chapman, S. S., Coriell, L. L., and Kowal, L. L. 1946 Streptomycin studies in tularemia. II Streptomycin therapy in white rats. (To be published).

42. Chapman, S. S., Coriell, L. L., and Nelson, W. E. 1946 Streptomycin studies in tularemia. III Streptomycin therapy in monkeys. (To be published).

43. Reed, L. J., and Muench, H. 1938 A simple method for estimating fifty per cent endpoints. Am. Jour. Hyg., 27, 493-497.

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

45. Snyder, T. L, Penfield, R. A., Engley, F. G. Jr., and Creasy, J. B. 1946 Cultivation of Bacterium tularense in peptone media. Proc. Soc. Exp. Biol. and Med., 63, 26-30.

46. Mills, R. C. Unpublished material.