CERTAIN IMMUNOLOGICAL RESPONSES OF WILD RODENTS AND LABORATORY ANIMALS FOLLOWING CHALLENGE WITH *PASTEURELLA TULARENSIS*

by

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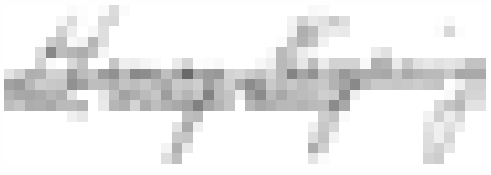

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I. INTRODUCTION

Pasteurella tularensis has long been known to be a disease agent of animals which can be transmitted to domestic animals and humans by contact with infected animals or by ectoparasite vectors. The incidence and distribution of Past. tularensis in wildlife have been a subject of many extensive surveys in various parts of the world. In making these surveys the primary methods of detecting past or present infection has been by the use of the tube agglutination test or by isolation of the Past. tularensis from infected host animals. The Ascoli type of test and the tularemia skin test have had very limited application. There have been no published experimental data to date related to the serological and hypersensitivity responses of wild rodents experimentally infected in the laboratory with Past. tularensis.

Diagnosis of tularemia in humans is readily accomplished by the tube agglutination test of Francis and Evans (1926), and by the tularemia skin test of Foshay (1932). The specificity of these tests has been studied in detail by many workers (Ransmeier, 1941, and Beerman and Ingraham, 1950). There have been no published data of experimental studies designed to study the nature of the hypersensitive state that is present following infection with Past. tularensis.

Preliminary studies in this laboratory suggested that wild rodents and guinea pigs may develop a hypersensitive state following a sublethal infection with Past. tularensis, which could be detected by a skin reaction. It was also found that rodents having recovered from a challenge with Past.

tularensis produced an antibody titer that would fix complement in the presence of a soluble polysaccharide extracted from Past. tularensis.

Because there has been no previous study of the serological and hypersensitivity responses of wild rodents experimentally challenged with Past. tularensis, these problems were considered to be of enough importance to attempt a detailed study. This investigation was intended to determine the agglutinin, complement fixing antibody, and cutaneous hypersensitivity response of rodents following survival of challenge with Past. tularensis. The characterization of the hypersensitive state following infection with Past. tularensis was studied in guinea pigs. A preliminary study of the inflammatory response following tularemia skin testing with Past. tularensis challenged rodents was also undertaken.

II. LITERATURE REVIEW

The etiological agent of tularemia, Pasteurella tularensis, Breed et al, (1957), was first cultured from the carcass of a ground squirrel (Citellus beecheyi), by McCoy (1911, 1912) during an epizootic in Tulare County, California. Since this original isolation of Past. tularensis, it has been demonstrated to be enzootic and epizootic in a wide range of mammalian fauna throughout the Northern Hemisphere. Burrows et al, (1945) have reviewed the reports of the isolation of Past. tularensis from a large number of wild animals including many species of rodents.

Since the review by Burrows et al, (1945) there have been numerous articles published concerning the enzootic and epizootic occurrence of tularemia in wildlife populations. These publications have been reviewed by Marchette et al, (1961). The occurrence of tularemia infections in humans has been reviewed by Francis (1942), and Fields (1949).

A. Immunological Responses of Animals and Humans Resulting from Pasteurella tularensis infection.

The diagnosis of naturally occurring tularemia in humans is usually made by a combination of clinical, epidemiologic, and serologic evidence. Past. tularensis is rarely recovered unless selective media and/or animal inoculations are routinely employed in suspected cases.

Specific diagnosis has centered around the agglutination reaction because of ready availability of the antigen and simplicity of performance of the test, although numerous other tests have been developed and have been used with varying degrees of reliability.

Friedewald and Hunt (1939) and Foshay (1948) have stated that the observation of an increase in the Past. tularensis agglutinating antibody titer of a patient's serum is the most reliable diagnostic test available for tularemia.

The observation of a single positive agglutination reaction does not indicate a present infection, but it is a reliable test for a previous infection. The agglutination reaction as presently in use was originally described by Francis and Evans (1926).

Like the skin test, the value and reliability of the agglutination reaction is significant in survey type work where the elapsed time between recovery and testing may be years. In humans, Ransmeier (1941) has shown that a positive tularemia titer may persist for the life of the individual.

Coriell et al, (1948) found the antibody response of the monkey to be similar to that of man, but no persistence studies were carried out beyond 32 days, at which time a maximum titer was still maintained. In a study on the persistence of the agglutination titers of sheep, Jellison and Kohls (1950) found that titers could be used to detect epizootics 57- months after they had occurred.

In immunization studies on the white mouse and white rat, Downs et al, (1947), and Downs and Buchele (1949), found that the agglutination titer of the immunized white mouse and white rat would decline from 1:640 to 1:20 in two months following injection of the immunizing material. The persistence of agglutinating titers in animals having been exposed to living virulent organisms has not been studied over prolonged periods. Past. tularensis agglutinin titers in guinea pigs, white rats, white mice, albino rabbits, and chickens repeatedly challenged with four Past. tularensis strains during a five week period ranged from 1:20 to 1:20,480 (Downs and Moody, 1955). The level of antibody appeared to be related to the strains of organisms used and the species of animals challenged.

The major disadvantage of the agglutination titer determination is the cross-reaction with Brucella abortus and Brucella meletensis as described

by Francis and Evans (1926). Ransmeier (1941), Lavergne et al, (1951), and Girard and Chevalier (1951) have reported that in most instances cross-agglutination will only occur if the anti-Past. tularensis titer is about 1:320 or above. A cross-reaction of tularemia serum with Proteus vulgarus OX_{19} was reported by Friedewald and Hunt (1939).

The slide agglutination test of Hull (1929) is of value in the rapid identification of Past. tularensis cultures. Conversely, known organisms may be employed for diagnostic purposes to identify serum agglutinins. In using this test for a rapid serological identification of sera containing Past. tularensis antibodies, Hull (1929) and Muraviev et al, (1943) found it to be sensitive to a titer as low as 1:15. Muraviev et al, (1943) recommended the use of this test as a general survey technique.

A recent development in serological diagnosis that may prove of value in the diagnosis of tularemia is the application of the hemagglutination reaction of Keogh (1948). He demonstrated that erythrocytes with adsorbed bacterial polysaccharide can be agglutinated by specific bacterial antibodies. A sero-diagnostic application of the hemagglutination reaction to detect Past. tularensis antibodies was utilized by Alexander et al, (1950), and further studied by Charkes (1959).

A lipopolysaccharide prepared from Past. tularensis proved satisfactory for use in the hemagglutination tests when adsorbed onto tannic acid-treated erythrocytes (Landy, 1954).

Larson (1947) obtained a thermostable antigen from carcasses of dead rabbits that could be used in a modified Ascoli type of test for tularemia. This type of test has been demonstrated by Larson (1951) to be valuable in the detection of a tularemia epizootic where only dead animals can be found.

The possible value of using a skin test as a diagnostic aid was mentioned by Wherry (1914) when studying tularemia conjunctivitis in a patient. Little notice was made of the comment until Foshay (1932, 1934) reported success in the practical application of a tularemia skin test in diagnosing human tularemia. Foshay found that heat killed and formalin killed antigens would elicit severe reactions in tularemia patients. However, by treating the formalin killed cells with nitrous acid or sodium ricinoleate, an antigen producing a less severe, but yet diagnostic reaction in tularemia patients was obtained. Volfertz (1934) developed a similar test for the early diagnosis of tularemia. A heat killed antigen was administered by making two superficial incisions in the skin and rubbing in a drop of the antigen. Lavergne et al. (1950) found that filtrates of five-day old broth cultures, emulsions of killed bacteria, and a dilution of endotoxin from Past. tularensis provoked a clear positive reaction in all patients with a positive tularemia titer. Drobinsky (1943) and Muraviev et al. (1943) in studying the reliability of the skin test found it to be positive in 94.7 to 98 per cent of proven tularemia cases.

Foshay (1934, 1948) and Drobinsky (1943) demonstrated that although Past. tularensis, Br. abortus and Br. melitensis share a common antigenic structure they will not cross-react with respect to the intradermal test. However, the matter of cross-reaction between these organisms in the skin test is still questionable (Lavergne, 1950). Doepfmer (1952) reported positive skin test reactions in patients with Lues latents and fungi responsible for the deep mycoses. A cross-reaction with the Ducrey bacilli was reported by Beerman (1950). Only in rare cases have positive reactions been observed in the normal person. These reactions reported in normals and as cross-reactions may be questioned because of lack of complete case histories of past illnesses of the individuals in question.

Foshay (1934) and Doepfmer (1952) found that the skin sensitivity following recovery of the patient from Past. tularensis infection would persist for as long as two to four years, and possibly longer. Hoshishima (1955) and Hoshishima et al, (1955) reported successful application of the tularemia skin test on a survey basis in the general population of Japan. Positive reactions were found in 16 of the 2,840 individuals tested.

The typical skin test reaction in humans has been well described. The reaction may vary, depending on the sensitivity of the patient and the type of antigen employed. Using a chemically treated type of antigen, Foshay (1932, 1934), Doepfmer (1952), and Lavergne (1950) observed a maximum reaction in 36 to 48 hours, consisting of erythema and soft edema measuring from one to six centimenters in diameter. Occasionally a papular response was observed. The reaction was found to subside in 5 days. Using the untreated, heat killed antigen, Volfertz (1934) observed a similar but more severe type of reaction accompanied by itching and formation of a brownish pigmentation that persisted for as long as 20 days after the reaction had subsided.

Considering the possible formation of agglutinins following a skin test, Friedewald and Hunt (1939) studied 12 tuberculosis patients with negative tularemia and brucellosis antibody titers. Intradermal inoculation of 0.05 ml of detoxified antigen adjusted to 50 ppm koalin turbidity was used. Two weeks later an agglutinin titer of 1:20 to 1:40 was observed in all patients. Two of the patients developed a mild positive reaction when given repeated intradermal tests. Friedewald and Hunt (1939) considered that confusion of this agglutinin response with the agglutinin response following tularemia to be most unlikely.

A second type of skin test using specific antisera to aid in the early diagnosis of tularemia was developed by Foshay (1935, 1936). Friedewald and Hunt (1939), and Lawless (1941) found this procedure of limited value because of the inconsistency ot the reaction.

There is little literature available concerning the application of the tularemia skin test to animals. Tumanskii and Kolensnikova (1939) have reported the use of a cutaneous allergic reaction in the diagnosis of tularemia in rodents. In studying the skin reaction in rabbits produced by intradermal inoculation of suspensions of killed Past. tularensis, Larson (1946), found that both normal and immunized rabbits produced erythema and edema following the inoculation of formalized suspensions of cells. By ether extraction or boiling the antigen for 30 minutes the reaction in normal rabbits was wholly or partially reduced. Immunized rabbits showed a lesser degree of skin reactivity suggesting that they are capable of partially neutralizing a skin toxic factor. Lavergne (1950) found the Past. tularensis immunized rabbit to be sensitive to an intradermal inoculation of a suspension of Past. tularensis cells grown for 6 weeks at 37 C, and heat killed at 100 C. The reaction produced was similar to that reported by Larson, but there was no mention of using any control animals. Kalitina (1958) has utilized the tularemia skin test in guinea pigs as an indication of the degree of immunity in tularemia.

B. The Nature of Cutaneous Hypersensitivity

Tularemia is among the group of chronic and granulomatous infectious diseases which include others such as tuberculosis, brucellosis, typhoid fever, glanders, vaccinia, mumps and lymphogranuloma venereum, in which delayed types of hypersensitivity play a predominant role in the host's responses to infection (Lawrence, 1956).

The technique of cellular transfer of delayed-type sensitivity from sensitized guinea pigs to normal guinea pigs was first accomplished by Bail (1910). In reviewing the literature, Kirchheimer (1947) found that only variable results could be obtained using the technique of Bail (1910). However, the efforts of Landsteiner and Chase (1942), and Chase (1945) demonstrated that leukocytes isolated from peritoneal exudates, spleen, lymph nodes, and whole blood of sensitized animals could be utilized to transfer specific cutaneous sensitivity to normal guinea pigs.

The fundamental work of Chase has been confirmed by Stavitsky (1948), and Metaxas and Metaxas-Buehler (1955). Kirchheimer et al, (1949) expanded this cellular transfer technique to demonstrate systemic as well as cutaneous sensitivity in recipient guinea pigs. The transfer of tuberculin sensitivity with almost pure suspensions of lymphocytes has been accomplished by Wesslen (1952). The possible role of granulocytes was shown to be unlikely by the work of Kirchheimer et al, (1951) when they were unable to transfer tuberculin sensitivity with these cells. Additional work of Chase (1953, 1955) has suggested that the capacity to transfer delayed-type sensitivity resided in the lymphocytes and monocytes.

Although by far the greatest volume of studies utilizing the technique of Chase (1945) has been in the study of tuberculin sensitivity, recent reports show the technique can be used to study other delayed sensitivities. Metaxas-Buehler (1951), and Carrers and Quatrifages (1952) have transferred sensitivity from brucella sensitive guinea pigs to normal guinea pigs. Salvin (1957) has adapted the cellular transfer technique to demonstrate the occurrence of an early phase of delayed-type of hypersensitivity prior to the development of the Arthus phenomenon in guinea pigs being sensitized with minute quantities of purified protein.

Lawrence (1949) has utilized the technique to transfer tuberculin sensitivity from man to man. Modeling his experiment after previous work in humans, Lawrence (1954) was able to demonstrate that sensitivity to streptokinase-streptodoinase and to streptococcal M-substance could be transferred from human to human by leukocytes collected from the peripheral blood. This work has been confirmed by Good and Varco (1955).

Attempts to transfer the delayed-type of hypersensitivity from one species to another has recently met with success. Schlange (1955) was able to transfer tuberculin sensitivity from children to guinea pigs with leukocytes, thus making it possible to detect tuberculin sensitive children without exposing them to tuberculin. Normal guinea pigs were sensitized to Mycobacteria lepraemurium by the injection of leukocytes collected from infected white rats (Wallace, 1957).

The necessity for viable cells in the transfer of delayed-type of sensitivity in the guinea pig and rabbit has been well established by Chase (1945). The possibility of transfer of delayed-type of sensitivity to tuberculin by sonically ruptured leukocytes has been suggested by Jeter (1954, 1957) and by Cummings et al, (1956). These results are strikingly different from the results obtained in man. The ease with which delayed-type of tuberculin and streptococcal sensitivity can be transferred in man by disrupted leukocytes has been well established by Lawrence (1955). Lawrence (1956) has also demonstrated this with regard to sensitivity to diphtheria toxoid. By utilizing sediments and extracts of disrupted leukocytes prepared by freezing and thawing, or by mechanical disruption, Friedman et al, (1957) have confirmed the observations of Lawrence (1955, 1956).

Passively sensitized guinea pigs have been shown to retain sensitivity for only 3 to 5 days (Chase, 1945, and Metaxas and Metaxas-Buehler, 1955). In man, recipients of leukocytes from sensitized individuals have remained

reactive for up to 6 months to 2 years following receipt of the sensitized cells (Lawrence, 1955). Chase (1958) has suggested that this apparent difference may not be as great as it first appeared. He has found that the persistence of sensitivity in the recipient animal may be greatly dependent on the number of cells transferred. Guinea pigs given cells of up to 10 times that required to sensitize the animal will greatly lengthen the persistence of the sensitivity in the recipient.

III. MATERIALS AND METHODS

A. Experimental Animals

The deer mice (*Peromyscus maniculatus*), grasshopper mice (*Onychomys leucogaster*), desert wood rats (*Neotoma lepida*), and bushy-tailed wood rats (*Neotoma cinerea*), used in this study were reared in the Ecological Research Faunal Colony, Dugway, Utah. At the beginning of the experimental studies these rodents were all sexually mature sub-adults. The Ord kangaroo rats (*Dipodomys ordii*) were field trapped and were mature adults of unknown ages.

Adult white mice, CFW strain; guinea pigs, Hartley strain; and albino rabbits, were obtained from the Department of Bacteriology, University of Utah. No attempts were made to segregate the experimental animals on the basis of sex.

B. Cultures

The *Pasteurella tularensis* strains used in this study were maintained on glucose cysteine blood agar (GCBA) slants and the *Brucella* species were maintained on Bacto-Tryptose Agar[1] slants. Fresh transfers of stock cultures were incubated for 24 to 48 hours at 37 C and stored at 4 C. Each stock culture was transferred monthly.

Pasteurella tularensis, Jap$_4$ strain, is a laboratory variant of a strain originally isolated from a human lymph node in Japan in 1926. It has been characterized as being only moderately virulent for white mice (Downs and Moody, 1955). This strain has also been shown to be of high virulence for grasshopper mice, of low virulence for kangaroo rats and deer mice, and avirulent for wood rats (Marchette et al, 1961).

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[1] Difco Laboratories, Inc., Detroit, Michigan.

Pasteurella tularensis, strain 38, was originally isolated by Francis in 1920 from a human lymph node, and subsequently became totally avirulent for all laboratory animals (Hesselbach and Foshay, 1945).

Pasteurella tularensis, strain 425 F_4G, was originally isolated from an engorged female tick, Dermacentor andersoni. It has been found to be virulent for white mice, moderately virulent for guinea pigs and avirulent for rabbits (Bell et al, 1955).

Pasteurella tularensis, strains NIIEG variant Blue and NIIEG variant Gray, were both of attenuated virulence for humans and laboratory animals (Kalitina, 1958).

Brucella suis strain DPG-2 was isolated from a jack rabbit, Lepus californicus, carcass, collected in Tooele County, Utah in 1955 (Stoenner et al, 1959). This organism was characterized by Stoenner et al, (1959) as being highly virulent for guinea pigs.

Brucella neotomae strain 60-152 was isolated from the tissue of a desert wood rat by Stoenner et al, in 1957.

The Past. tularensis strains, Jap_4, 38, and NIIEG variants were obtained from Dr. H. T. Eigelsbach, Fort Detrick, Maryland. The 425 F_4G strain of Past. tularensis was obtained from Dr. Cora Owen of the Rocky Mountain Laboratory, Hamilton, Montana. The Brucella strains were obtained from Dr. H. G. Stoenner, also of Rocky Mountain Laboratory.

Suspensions of cells of the strains used for the inoculation of animals were made in gelatinized saline (0.2% gelatin in 0.85% NaCl) from 24 hour slant cultures incubated at 37 C. A single sublethal inoculation of the suspended viable Past. tularensis or Brucella suis cells was routinely used to produce infection in experimental animals. Guinea pigs were inoculated subcutaneously in the right flank area.

C. Preparation of Antigens.

Heat-killed antigens were prepared from a 0.15 M saline suspension of strains Schu A, 38 and Jap_4 of Past. tularensis cultured at 37 C for 24 hours on glucose-cysteine-blood-agar (GCBA). The cells were washed twice, suspended at a final concentration of 5×10^8 cells per ml, and killed by incubating in a 60 C water bath for one hour. The Schu A preparation contained 0.229 mg N per ml, the 38 preparation contained 0.094 mg N per ml, and the Jap_4 preparation contained 0.216 mg N per ml as determined by micro-Kjedahl analysis.

A second suspension of Schu A prepared as described above was killed by the addition of formalin to a final concentration of 0.2%. This suspension was washed twice with 0.5 M saline and adjusted to contain 5×10^8 cells per ml. The final suspension contained 0.25 mg N per ml as determined by micro-Kjedahl analysis. This cell suspension was used for both skin testing studies and as the agglutinating antigen.

Polysaccharide from Past. tularensis was prepared according to the method of Nicholes (1946) and Alexander et al. (1950). A portion of lyopholized polysaccharide was reconstituted in distilled water and washed four times with equal volumes of chloroform. Following 15 minutes shaking, the polysaccharide in water-chloroform mixture was allowed to stand so that the water and chloroform would separate. There was no detectable emulsion of denatured protein formed. This portion was again lyopholized after the chloroform washing. Micro-Kjedahl analysis of both the chloroform treated and the non-chloroform treated polysaccharide yielded 4.6% nitrogen. A portion of the non-chloroform extracted polysaccharide was used as the complement fixing soluble antigen.

A Past. tularensis cell wall preparation was obtained from Dr. Larson (Ormsbee, 1955), Rocky Mountain Laboratory, Hamilton, Montana.

A lyophilized Past. tularensis Schu A sonic lysate was obtained from Dr. P. S. Nicholes, Department of Bacteriology, University of Utah. This preparation contained 0.074 mg of N per ml when 14.5 mg was dissolved in 25 ml of sterile distilled water.

The Brucellergen (Brucella Protein Nucleate) used in this study was purchased from Sharpe and Dohme, Philadelphia, Pennsylvania (Huddleson et al. 1943).

Skin testing of rodents was routinely performed by intradermal injection of 0.05 ml of the appropriate antigen in a region of the abdomen from which the hair had been removed with electric clippers. The injection sites were observed intermittently during the first hour following administration of the antigen, then at intervals of 2, 4, 6, and 24 hours; then daily for 3 to 7 days.

D. Serology

To collect serum for serological studies, guinea pigs and wood rats were bled by cardiac puncture. All other rodents were bled by severing the subclavian vein and collecting free flowing blood in a small test tube. Serum was separated from this blood and stored at -20 F until the titer for Past. tularensis agglutination or complement fixing antibodies was determined.

The agglutinin titers were determined by the tube agglutination procedure of Francis and Evans (1926). The antigen used consisted of formalin killed Past. tularensis, strain Schu A cells, prepared as described above in Section C. Phenol was added to a final concentration of 0.5% as a preservative.

The complement fixation test was carried out according to the Communicable Disease Center virus technique with minor but modifications.

Past. tularensis polysaccharide at a concentration of 3.5 micrograms per ml was used as the antigen.

E. Serum Passive Transfer of Cutaneous Hypersensitivity

To test for the presence of the Arthus type of reaction in the tularemia skin test reaction, the following methods were used. Serum samples from the sensitized guinea pigs were transferred to the recipient guinea pigs by intradermal injection of 0.1 ml serum, into an area of the back of the animal, which had been shaved with electric clippers. Irritation of the skin was avoided as much as possible.

Each serum sample was injected in duplicate into the back of each of two young guinea pigs, weighing 300 to 500 grams. Twenty-four hours after transfer of the serum, one site of the injection area was skin tested for cutaneous sensitivity with the heat killed Schu A suspension and the duplicate on the same guinea pig was skin tested with the polysaccharde.

To quantitate this serum transfer test, infected recovered guinea pigs were exsanguinated by cardiac puncture and serum collected for passive transfer studies. The serum thus collected was used in the adaptation of a quantitative precipitin test using Past. tularensis polysaccharide as the soluble antigen.

A preliminary experiment to obtain the optimal concentrations of polysaccharide to precipitate the antibody was carried out according to Kabat and Meyer (1958), with but one modification. All procedures were carried out at 4 C.

Antibody nitrogen determinations were carried out according to Pressman (1943) using the phenol reagent as described by Folin and Ciocaltieu (1927). The results of the precipitin antibody determination from the analysis with the phenol reagent is originally expressed in ug tryrosine per ml of serum. Following the recommendation of Kabat and

Meyer (1958) a sample of specific polysaccharide precipitate was analyzed by both the phenol reagent and standard micro-Kjedahl methods. Using triplicate samples it was found that Past. tularensis polysaccharide antibody nitrogen in mgs equalled 2.95 times the ug tyrosine content of the precipitate.

F. Cellular Passive Transfer of Cutaneous Hypersensitivity

Adult, albino guinea pigs were sensitized by a single subcutaneous inoculation of 10^9 living Past. tularensis, strain Jap_4 cells. All such sensitizing inoculations were made into the loose skin over the back of the neck so that the resulting lesion would be remote from, and not interfere with, the pr eparations of peritoneal exudates. Suspensions of peritoneal exudates. Suspensions of peritoneal exudate cells were collected from challenged guinea pigs, using the method of Tremaine (1957). The cells obtained from four to six sensitized guinea pigs by this method were pooled and immediately injected subcutaneously into the inguinal area of a normal guinea pig weighing 200 to 500 grams. At appropriate intervals, recipient guinea pigs were tested by intradermal injections of the Past. tularensis, Schu A, heat killed suspension and with the polysaccharide preparation. The skin sited test was observed at various intervals for the first 4 hours, and at 24 and 48 hours after skin testing. Appropriate control guinea pigs were used.

IV. RESULTS

A. Cutaneous Response of Pasteurella tularensis Challenged Animals to Intradermal Injection of Various Pasteurella tularensis Preparations

1. Deer mice.

Deer mice were observed to elicit a cutaneous response to intradermal injection of several Past. tularensis preparations three weeks after challenge with 10^3 viable Past. tularensis strain Jap_4 organisms. From the summarization of these responses as presented in Tables 1 and 2, it is readily evident that several different preparations possessed the capacity to stimulate a cutaneous response in the challenged deer mice.

The positive cutaneous response of challenged mice to all of the preparations was a typical circular area of induration, rarely accompanied by erythema or edema. Twenty-four hours after testing, the typical cutaneous reactions were generally unapparent or similar to the response of the control animals, but by 48 hours the response had become maximal in the challenged animals and had disappeared in the control animals. The peak reaction had begun to decrease in intensity by 72 hours and gradually subsided until no apparent response could be observed by the 5th to 7th day following injection of the skin test dose. The various heat killed suspensions of Past. tularensis elicited cutaneous responses of a similar magnitude. Intradermal injection of ten-fold serial dilutions of the Schu A heat killed preparation resulted in a decreasing number of positive reactions in the challenged animals. The formalin killed Schu A and the polysaccharide prepared from this strain also induced a cutaneous response in challenged deer mice. In each case, with decrease in concentration of the Schu A preparation there was an apparent lessening in the intensity of the cutaneous response of the test animals. Deer mice tested

TABLE 1

The cutaneous responses of deer mice to intradermal injection of various Pasteurella tularensis preparations three weeks after challenge with 10^3 Past. tularensis Jap_4 cells.

Past. tularensis Preparation	Time after intradermal injection of antigen					
	24 hrs		48 hrs		72 hrs	
	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]
Heat killed 38	0/10	0	10/10	3	10/10	3
Heat killed Jap_4	4/10	3	10/10	4	10/10	4
Heat killed Schu A	5/10	3	10/10	5	10/10	4
" 1:10	1/10	3	3/10	4	3/10	3
" 1:100	0/10	0	1/10	3	0/10	0
Sonically ruptured Schu A	8/10	3	10/10	6	10/10	5
Formalin killed Schu A	8/17	3	14/17	4	14/17	3
Polysaccharide	2/10	3	10/10	4	10/10	4
Saline	0/10	0	0/10	0	0/10	0

1 Animals developing an observable cutaneous response.
2 Expressed as average diameter of reactions in mm.

TABLE 2
The cutaneous responses of normal deer mice to intradermal injection of various Pasteurella tularensis preparations.

Past. tularensis Preparation	Time after intradermal injection of antigen					
	24 hrs		48 hrs		72 hrs	
	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]
Heat killed 38	2/10	2	0/10	0	0/10	0
Heat killed Jap$_4$	3/10	2	0/10	0	0/10	0
Heat killed Schu A	3/10	2	0/10	0	0/10	0
" 1:10	0/10	0	0/10	0	0/10	0
" 1:100	0/10	0	0/10	0	0/10	0
Sonically ruptured Schu A	4/10	2	0/10	0	0/10	0
Formalin killed Schu A	0/10	0	0/10	0	0/10	0
Polysaccharide	0/10	0	0/10	0	0/10	0
Saline	0/10	0	0/10	0	0/10	0

1 Animals developing an observable cutaneous response.
2 Expressed as average diameter of reactions in mm.

with the sonically ruptured Past. tularensis Schu A preparation developed the greatest cutaneous response.

2. Guinea Pigs.

Guinea pigs that were skin tested with various preparations three weeks after subcutaneous challenge with 10^3 Past. tularensis Jap_4 organisms developed a cutaneous response different than that of the challenged deer mice. Intradermal injection of 0.05 ml of the heat killed Schu A suspension evoked an erythema ranging from 15 mm to 20 mm in diameter, with central necrosis and/or formation of vesicules of 3 mm to 10 mm in diameter. Similar, if not identical, responses were also elicited following skin test with other heat killed Past. tularensis strains, as well as with the polysaccharide, Table 3. The injection site gradually began to redden about 30 minutes after injection of the test antigen and after 2 hours nearly all animals tested with the heat killed antigen developed an edematous area averaging 6 mm in diameter with very little erythema. Injection of polysaccharide induced a strong erythema averaging 10 mm in diameter with central vesiculation within 2 hours. After nine hours central vesiculation and hemorrhage appeared at the site of the injection of both preparations and the overall diameter of the reaction increased to 10 mm to 20 mm in diameter. These reactions continued to increase in intensity, reaching a peak by 24 hours. Thereafter, the reactions subsided and within 4 days the injured tissues sloughed and healing progressed. By the 7th to 9th day only scar tissue remained.

Table 4 summarizes the observations of the development of the cutaneous response of guinea pigs when skin tested three weeks after challenge with 10^3 Jap_4 strain organisms.

TABLE 3

The cutaneous response of guinea pigs 24 hours after intradermal injection of various Pasteurella tularensis preparations three weeks after challenge with 10^3 Past. tularensis Jap_4 organisms.

Past. tularensis Preparation	Challenged		Normal	
	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]
Heat killed 38	7/7	20	3/10	2
Heat killed Jap_4	7/7	20	4/10	2
Heat killed Schu A	23/23	25	4/10	3
Formalin killed Schu A	6/6	10	1/2	2
Polysaccharide	17/17	20	0/10	0
Cell wall	0/2	0	0/2	0
Saline	0/2	0	0/2	0

1 Animals developing an observable cutaneous response.
2 Expressed as average diameter of reactions in mm.

TABLE 4

Observations on the development of the cutaneous response of skin tested guinea pigs three weeks after subcutaneous challenge with 10^6 Pasteurella tularensis Jap_4 cells.

Hours after skin testing	Past. tularensis Preparation	Reactors[1] per total	Reactions[2]	Description of reaction
1/4	Heat killed Schu A	0/10	0	
	Polysaccharide	0/10	0	
1/2	Heat killed Schu A	1/10	3	Slight erythema
	Polysaccharide	2/10	3	Slight erythema
2	Heat killed Schu A	1/10	6	Slight erythema
	Polysaccharide	9/10	10	Slight erythema
9	Heat killed Schu A	10/10	12	Erythema with central hemorrhage
	Polysaccharide	10/10	17	Erythema with central hemorrhage
24	Heat killed Schu A	10/10	25	Erythema with central hemorrhage
	Polysaccharide	10/10	22	Erythema with central hemorrhage
48	Heat killed Schu A	10/10	19	Erythema with central hemorrhage
	Polysaccharide	10/10	18	Erythema with central hemorrhage

1 Animals developing an observable cutaneous response.
2 Expressed as average diameter of reactions in mm.

3. Rabbits.

Six rabbits challenged with 10^9 Past. tularensis strain 425 F_4G cells also elicited a cutaneous response following intradermal injection of 0.05 ml of various preparations, Table 5. Each challenged rabbit was tested by intradermal injection of several preparations. The cutaneous response of challenged rabbits was very similar to the response observed in the sensitized guinea pigs, in contrast to that seen in deer mice. Twenty-four hours after skin testing, rabbits developed an erythematous zone up to 20 mm in diameter. However, unlike the response of guinea pigs, that of the rabbits continued to develop, reaching a peak of 25 mm diameter at 48 hours. There was an apparent lack of edema in the response of the rabbits to all preparations employed.

In addition to using the polysaccharide and the heat killed Schu A suspension described above, both were further diluted 1:10 and 1:100 in sterile saline. Two rabbits challenged three weeks previously with 10^9 Past. tularensis 425 F_4G and one normal rabbit were skin tested with these dilutions. The response evoked by the diluted preparations is summarized in Table 5. It is apparent that intradermal injection of as little as 9.0 ug would not. The Schu A heat killed preparation at a concentration of 0.11 ug N per ml elicited a response in the challenged rabbits. The diluted polysaccharide and the Schu A cell suspension elicited no visible response in normal rabbits.

The Past. tularensis cell wall preparation obtained from Dr. Larson caused an erythema 5 mm in diameter in challenged rabbits, but no response in normal rabbits.

4. White Mice.

Three weeks after intraperitoneal challenge with 10^3 viable Past. tularensis strain Jap_4 cells, white mice were also found to have developed

TABLE 5

Cutaneous response of rabbits 48 hours after intradermal injection of various Pasteurella tularensis preparations three weeks after challenge with 10^9 Past. tularensis 425 F_4G organisms.

Past. tularensis Preparations	Challenged		Normal	
	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]
Heat killed Schu A	2/2	22	0/1	0
Heat killed 1:10	2/2	12	0/1	0
Heat killed 1:100	1/2	11	0/1	0
Polysaccharide	2/2	23	0/1	0
Polysaccharide 1:10	2/2	5	0/1	0
Polysaccharide 1:100	0/2	0	0/1	0
Cell wall	2/2	4	0/1	0
Saline	0/2	0	0/1	0

1 Animals developing an observable cutaneous response.
2 Expressed as average diameter of reaction in mm.

degrees of cutaneous hypersensitivity to various Past. tularensis preparations. All of the ten white mice skin tested with the heat killed and the formalin killed Schu A suspensions elicited cutaneous responses. The response was similar to that of the deer mice, both in the apparent type of response and in the time interval of progressive development of the response, following the skin test injection. None of the normal white mice elicited a significant response following intradermal injection of either preparation.

B. The Cutaneous Responses of Certain Rodents to the Intradermal Injection of Heat Killed Schu A Cells, Following Challenge with Various Pasteurella tularensis Strains.

1. Jap_4 Strain.

In a study to determine the effect of varying challenge dosages on the development of cutaneous sensitivity, groups of deer mice, Ord kangaroo rats, desert wood rats, bushy-tailed wood rats, and grasshopper mice were challenged subcutaneously with ten-fold serial dilutions of Past. tularensis strain Jap_4.

The results of this study are presented in Table 6. It is apparent that as little as ten viable organisms of the Jap_4 strain will sensitize the majority of the deer mice and grasshopper mice tested. In Ord kangaroo rats, one of four became sensitive after challenge with 10^2 Jap_4 cells, and the majority of animals receiving 10^3 or greater numbers of cells became sensitive to skin test antigen.

The cutaneous response to intradermal injection of heat killed Schu A cell preparation of the other challenged rodent species was similar to that described in deer mice. A possible exception to this is the grasshopper mouse, in which the appearance of erythema and edema within 24 hours was common among the challenged and skin tested animals. At 48 hours, the

TABLE 6

Cutaneous response of certain wild rodents to heat killed Schu A cells three weeks after subcutaneous challenge with 10-fold serial dilutions of *Pasteurella tularensis* strain Jap_4.

Chall. Dose	Deer Mice		Grasshopper Mice		Ord kangaroo rats		Desert wood rats		Bushy-tailed wood rats	
	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]
10^1	4/7	4	7/7	6						
10^2	26/30	4	10/10	6	1/4	6				
10^3	18/30	5	7/7	6	3/6	5				
10^4	27/28	5	7/9	7	5/8	6	8/8	6		
10^5	16/17	5	3/3	7	2/3	6	16/16	6	1/2	6
10^6	8/8	6			1/1	7	16/16	6		
10^7							14/16	6		
10^8							8/8	6	3/3	7
Control	0/40	0	0/20	0	0/12	0	0/6	0	0/3	0

[1]Animals developing an observable cutaneous response.
[2]Expressed as average diameter of reactions in mm.

lesion had become indurated and resembled the typical response of the other rodents.

The similarities of the overt appearance of the cutaneous response of challenged deer mice and grasshopper mice may be seen by examining Figs. 1 and 2.

The wood rat, though highly resistant to Past. tularensis Jap_4 organisms, readily became sensitive if inoculated with 10^4 or more organisms. All but two of the 64 animals challenged with 10^4 to 10^8 cells became positive reactors to heat killed Schu A skin test antigen.

The bushy-tailed wood rat also became sensitized to an intradermal injection of heat killed Schu A cells following challenge with 10^5 and 10^8 Jap_4 cells. Though only eight animals were available for testing, four of the five animals challenged developed a positive cutaneous response. In all Jap_4 challenged animals, there was no apparent differences in the magnitude of the cutaneous response elicited by intradermal injection of the Schu A preparation.

2. NIIEG Strain.

Deer mice were also challenged subcutaneously with varying numbers of both of the varieties of the NIIEG strain of Past. tularensis.

Varying challenge of the NIIEG strain in deer mice appeared to have little if any effect upon the intensity of the cutaneous response of the surviving animals, Table 7. The positive responses were similar to those previously described as to both the time of peak response and the overt appearance of the reaction. The NIIEG variant Blue appeared to be very much like the Jap_4 strain, in that as few as 10 organisms induced cutaneous sensitivity to subsequent skin testing. However, it appeared that the inoculation of at least 10^5 organisms of the NIIEG variant Gray were essential to establish cutaneous sensitivity in the deer mice.

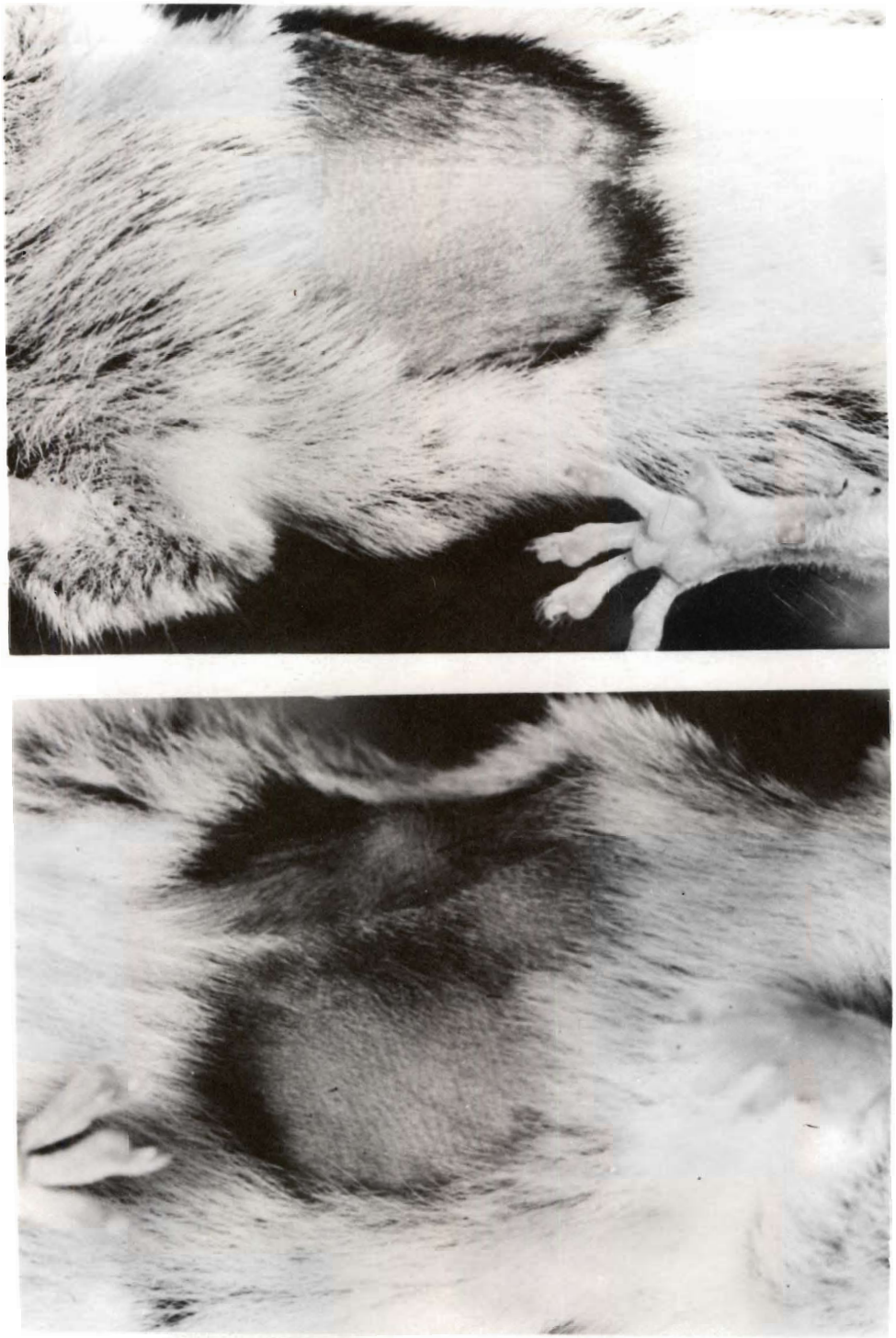

Fig. 1. Cutaneous reaction of deer mice, Peromyscus maniculatus, following intradermal injection of a heat-killed suspension of Pasteurella tularensis, strain Schu A. (Upper) Control deer mouse. (Lower) Past. tularensis challenged deer mouse. Observations 48 hours after skin testing.

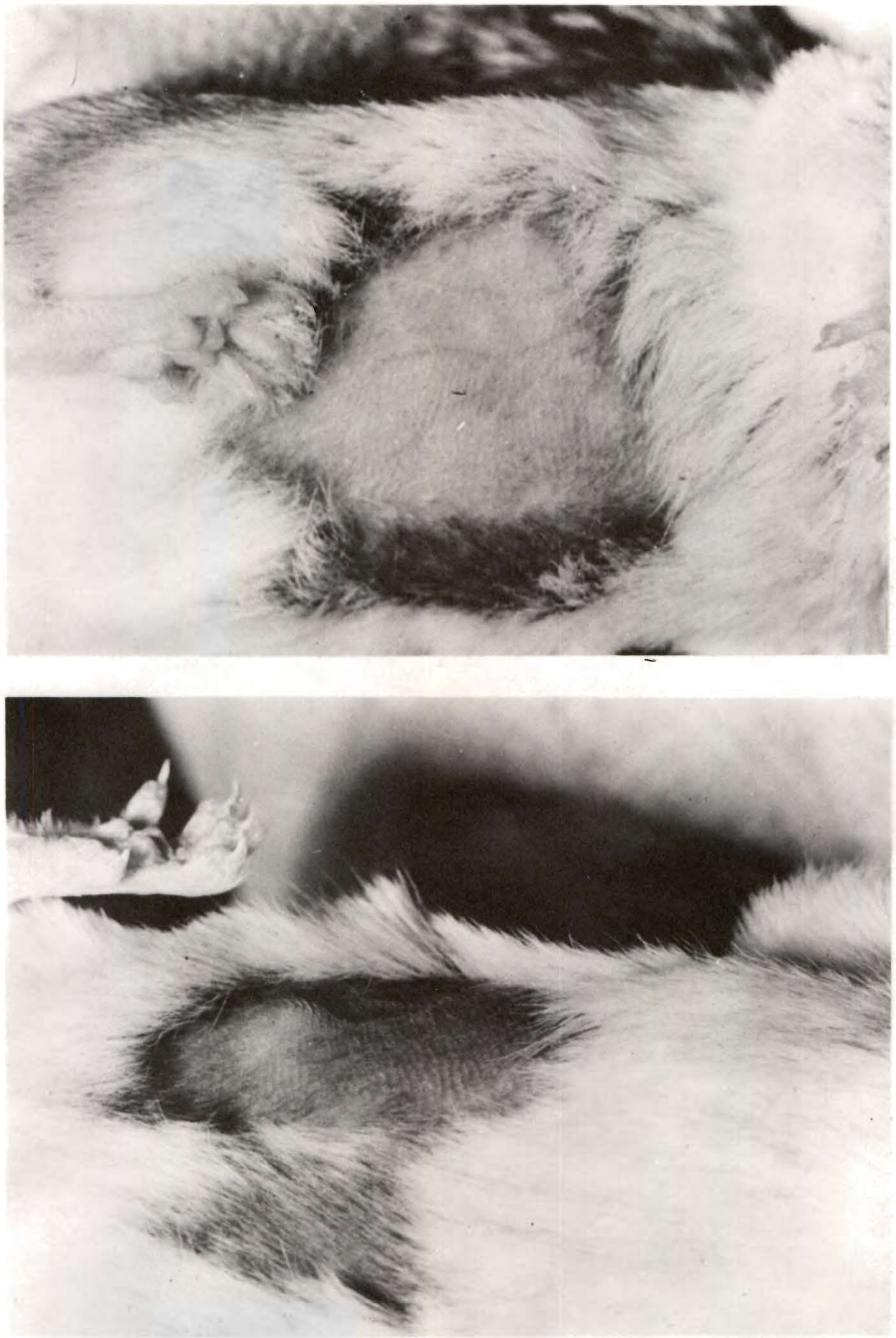

Fig. 2. Cutaneous reaction of grasshopper mice, Onychomys leucogaster, following intradermal injection of a heat-killed suspension of Pasteurella tularensis, strain Schu A. (Upper) Control grasshopper mouse. (Lower) Past. tularensis challenged deer mice. Observations 24 hours after skin testing.

TABLE 7

The cutaneous response of deer mice and wood rats to intradermal injection of heat killed Schu A cells three weeks after challenge with the Blue and the Gray variants of the NIIEG strain of Past. tularensis.

Challenge Dose	Challenge Strain			
	NIIEG (var Blue)		NIIEG (var Gray)	
	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]
	Deer Mice			
10^1	1/1	6	0/4	0
10^2	2/2	4	0/3	0
10^3	2/2	3	0/4	0
10^4	2/2	3	0/4	0
10^5	2/2	5	3/4	3
10^6			3/4	3
10^7	1/1	3	4/4	4
10^8			4/4	4
	Desert wood rat			
10^6	2/2	6	2/2	4

1 Animals developing an observable cutaneous response.
2 Expressed as average diameter of reaction in mm.

Desert wood rats were challenged with 10^6 organisms of both NIIEG variants (Table 7). When the surviving animals were skin tested with the heat killed Schu A antigen, both desert wood rats challenged with NIIEG variant Blue and two of the three challenged with NIIEG variant Gray, became sensitive to cutaneous injection of the heat killed Schu A cells.

C. The Persistence of Cutaneous Sensitivity in Certain Rodents Challenged with Pasteurella tularensis Jap$_4$ Cells.

The persistence of cutaneous sensitivity to intradermal injection of heat killed Schu A cells in 125 deer mice, 33 desert wood rats, and 21 Ord kangaroo rats following subcutaneous challenge with 10^3 Past. tularensis strain Jap cells has been examined, Table 8. In deer mice, eight of ten animals were sensitive to a skin test dose as early as one week, and 15 of 20 were skin positive two weeks following challenge. Between the third and 38th week following challenge, six to ten deer mice skin tested at varying intervals were positive. None of eight tested ten weeks leter were positive. Four of seven animals skin tested 53 weeks after challenge elicited a positive response.

Of the challenged Ord kangaroo rats, all that were skin tested through the 25th week following challenge were positive. Two of four Ord kangaroo rats skin tested at 40 weeks were positive.

All but one of the challenged desert wood rats skin tested with the heat killed Schu A cell preparation at intervals through 25 weeks following challenge were positive. There was no apparent decrease in the intensity of the reaction in any test group whether tested 1 week or 53 weeks after challenge. Throughout this experiment none of the 96 deer mice, 15 Ord kangaroo rats, and 17 desert wood rats utilized as control animals showed a positive response to the skin test antigen.

TABLE 8

Cutaneous sensitivity persistence in deer mice, Ord kangaroo rats, and desert wood rats challenged with 10^3 *Pasteurella tularensis* Jap_4 cells.

Time in Weeks	Deer mouse		Ord kangaroo rat		Desert wood rat	
	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]	Reactors[1] per total	Reactions[2]
1	8/10	5				
2	15/20	5				
3	9/10	5			7/7	6
6	8/10	4	5/5	5	4/4	6
7	7/10	6			5/5	6
12	10/10	4	4/4	5	4/5	7
18	10/10	4	4/4	5	6/6	6
25	10/10	5	4/4	5	6/6	6
31	6/10	4				
40			2/4	5		
48	0/8	0				
53	4/7	4				
Controls	0/96	0	0/15	0	0/17	0

[1]Animals developing an observable cutaneous response.
[2]Expressed as average diameter of reactions in mm.

D. Specificity of the Tularemia Skin Test in Rodents.

The possible presence of cross reactions of the tularemia skin test in rodents challenged with Br. suis was studied. Three weeks following challenge with Br. suis mice were skin tested with 0.05 ml of Brucellergen and heat killed Schu A antigen. None of the animals reacted to either antigen.

The observations of Mika et al. (1958) prompted an additional experiment with the wild Br. suis strain. It was observed by Mika that guinea pigs infected with Br. suis developed a positive skin test when a formalin killed suspension of Past. tularensis was injected intradermally after the 34th day following challenge.

Two hundred deer mice were challenged with 0.2 ml of a 10^{-1} dilution of a suspension of Br. suis washed from 24-hour tryptose agar slant culture of organisms. Of the challenged deer mice, 168 died within 36 days. Twenty-one deer mice, all with scar tissue 0.5 mm to 1 mm in diameter at the site of Br. suis inoculation were skin tested 42 days after challenge. Each deer mouse was skin tested with 0.05 ml of the heat killed Schu A cell suspension and 0.05 ml of Brucellergen. The heat killed Schu A cells suspension and Brucellergen elicited no response in the deer mice.

In addition to utilizing the Br. suis strain deer mice were also skin tested following challenge with Br. neotomae. Of 72 deer mice challenged with 10^1 to 10^8 organisms, two elicited a positive cutaneous response after intradermal injection of the heat killed Schu A antigen four weeks following challenge. One animal that received 10^2 organisms developed an induration measuring 5 x 5 mm 48 hours after skin testing with the Schu A heat killed suspension. The remaining 70 rodents failed to exhibit any response following intradermal injection of the antigen. These animals were not tested with Brucellergen.

E. Tularemia Skin Testing of Rodents Trapped Alive in an Area where a Tularemia Epizootic in Jack Rabbits had Occurred.

During the month of June 1958, a tularemia epizootic in the rabbit population in North Skull Valley, Utah occurred. Past. tularensis subsequently was isolated from one cottontail rabbit carcass and from one jack rabbit carcass taken from this area (Marchette et al. 1961). During June and July, 1958, 213 rodents, including eight species, were collected alive and skin tested with the heat killed Schu A strain, Table 9.

An additional 15 rodents of the above species were found dead in the traps, or died before they could be tested for cutaneous sensitivity to the Schu A heat killed antigen. These were autopsied and the spleens removed and homogenized. Past. tularensis could not be recovered from the homogenate by culture on glucose cysteine blood agar, or by deer mouse challenge. Ectoparasites were removed from all trapped rodents, titrated and inoculated into guinea pigs. Past. tularensis was not receovered by this method. One week following skin test the rodents were bled. Agglutinin titers ranged from negative to 1:80.

One hundred and twenty-eight control rodents were obtained from laboratory stock or by field trapping in areas remote from the epizootic area. A single intradermal injection of the heat killed Schu A cell suspension in these animals is summarized in Table 10. From this work it would appear that normal deer mice, grasshopper mice, Ord kangaroo rats, and wood rats may respond to a single intradermal injection of the heat killed Schu A antigen by developing complement fixing antibody titers and agglutination antibody titers ranging from negative to 1:160.

In conjunction with this study, deer mice, Ord kangaroo rats, and desert wood rats challenged three to four weeks previously with 10^3 Jap_4 strain Past. tularensis cells were skin tested with the heat killed Schu A

TABLE 9

The results of tularemia skin testing of rodents from an area where a tularemia epizootic had occurred in jack rabbits. Rodents from areas 20 miles or more distant from the epizootic area were collected for controls.

Rodent species	Rodents from tularemia epizootic area	Rodents from distant areas
	Reactors[1] per total	Reactors[1] per total
Dipodomys microps Chisel-toothed kangaroo rat	0/134	0/24
D. ordii Ord kangaroo rat	0/12	0/24
Peromyscus maniculatus Deer mouse	0/21	0/8
Citellus leucurus Antelope ground squirrel	0/22	0/12
Mus musculus House mouse	0/1	
Reithrodontomys megalotis Harvest mouse	0/4	0/23
Perognathus parvus Great Basin pocket mouse	0/1	0/13
Onychomys leucogaster Grasshopper mouse	0/1	
Perognathus formosus Long-tailed pocket mouse		0/6
Peromyscus crinitus Canyon mouse		0/18

[1]Animals developing an observable cutaneous response.

TABLE 10

Pasteurella tularensis agglutination and complement fixation titers of certain normal rodents one week after intradermal injection of the heat killed Schu A cell suspension.

Rodent species	Number of sera samples pooled	Titers	
		Agglutination[1]	Complement fixing[2]
Peromyscus maniculatus Deer mouse	10	40	20
	10	160	160
	10	0	0
	10	0	20
	10	0	40
	10	0	0
	10	80	20
	10	80	0
Onychomys leucogaster Grasshopper mouse	10	80	80
	10	160	40
Dipodomys ordii Ord kangaroo rat	1	40	80
	1	80	0
	1	0	0
	1	0	0
	1	20	0
	1	80	0
	1	160	0
	1	20	0
Neotoma lepida Desert wood rat	1	0	0
	1	0	0
	1	0	0
	1	0	0
	1	0	20
	1	0	40
	1	80	0
	1	20	0
	1	40	0
	1	40	0
	1	0	0
	1	40	0
	1	40	0
	1	0	0
	2	80	0
	2	0	0
	3	0	20

[1]Titers shown as the reciprocal of the highest serum dilution agglutinating *Past. tularensis* antigen to +2 or greater.

[2]Titers shown as the reciprocal of the highest serum dilution showing 50% lysis of sensitized sheep red blood cells.

cell suspension and bled one week later. The serum collected from this blood was then studied for Past. tularensis agglutinating and complement fixing antibodies. The results of this study are summarized in Table 11. It is evident that in general the antibody titers of the skin tested challenged animals were much higher than those of the skin tested normal animals. From these studies it appears that agglutination and complement fixing antibody titers of sera taken from rodents one week after skin test would yield only questionable evidence as to past Past. tularensis infection.

F. Histological Observation of the Tularemia Skin Test in Deer Mice and Grasshopper Mice.

Deer mice and grasshopper mice were tested for cutaneous sensitivity with heat killed Schu A cell suspension three weeks following challenge with sublethal doses of Past. tularensis strain Jap_4. The section of the tissue containing the resulting cutaneous lesions was removed from both normal and challenge animals 24 and 48 hours after test. These tissues were placed immediately into vials containing Bouin's tissue fixative. Tissue sections 7 microns thick were prepared and stained by the hematoxylin and eosin technique. It was apparent that sensitized rodents suffered an acute, local inflammatory response with edema following intracutaneous injection of the heat killed Schu A cells, Figures 3 and 4. In normal rodents only a mild, transitory inflammatory response was evident.

TABLE 11

Pasteurella tularensis agglutination and complement fixation titers of certain Jap4 challenged rodents on week after skin testing with the heat killed Schu A suspension.

Rodent species	Number of sera samples pooled	Titers	
		Agglutination[1]	Complement fixing[2]
Peromyscus maniculatus			
Deer mouse	10	640	640
	10	1280	640
	10	640	320
	10	640	320
	20	640	640
Dipodomys ordii			
Ord kangaroo rat	8	640	160
	6	640	320
	4	320	320
	3	1280	40
	1	320	640
	1	640	20
	1	40	160
	1	160	40
	1	80	80
	1	640	80
Neotoma lepida			
Desert wood rat	8	20	320
	8	20	160
	8	160	160
	1	320	40
	1	80	20
	1	160	40
	1	640	160
	1	320	40
	1	640	160
	1	320	80

[1]Titers shown as the reciprocal of the highest serum dilution agglutinating Past. tularensis antigen to +2 or greater.

[2]Titers shown as the reciprocal of the highest serum dilution showing 50% lysis of sensitized sheep red blood cells.

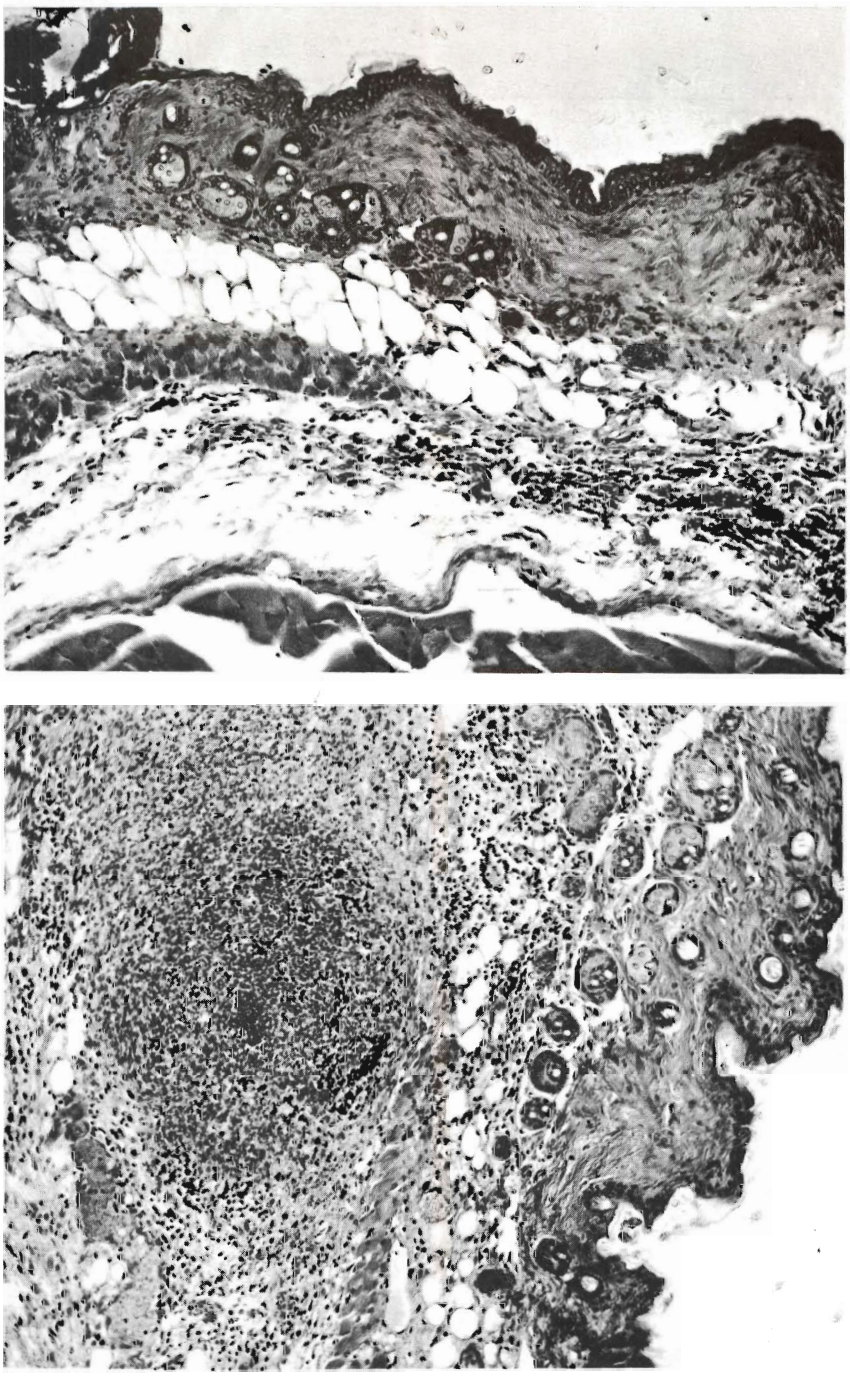

Fig. 3. Cutaneous reaction of deer mice, Peromyscus maniculatus, following intradermal injection of a heat-killed suspension of Pasteurella tularensis, strain Schu A. (Upper) Control deer mouse. (Lower) Past. tularensis challenged deer mouse. Observations 48 hours after skin testing. Bouin's fixation; haematoxylin and eosin. X100.

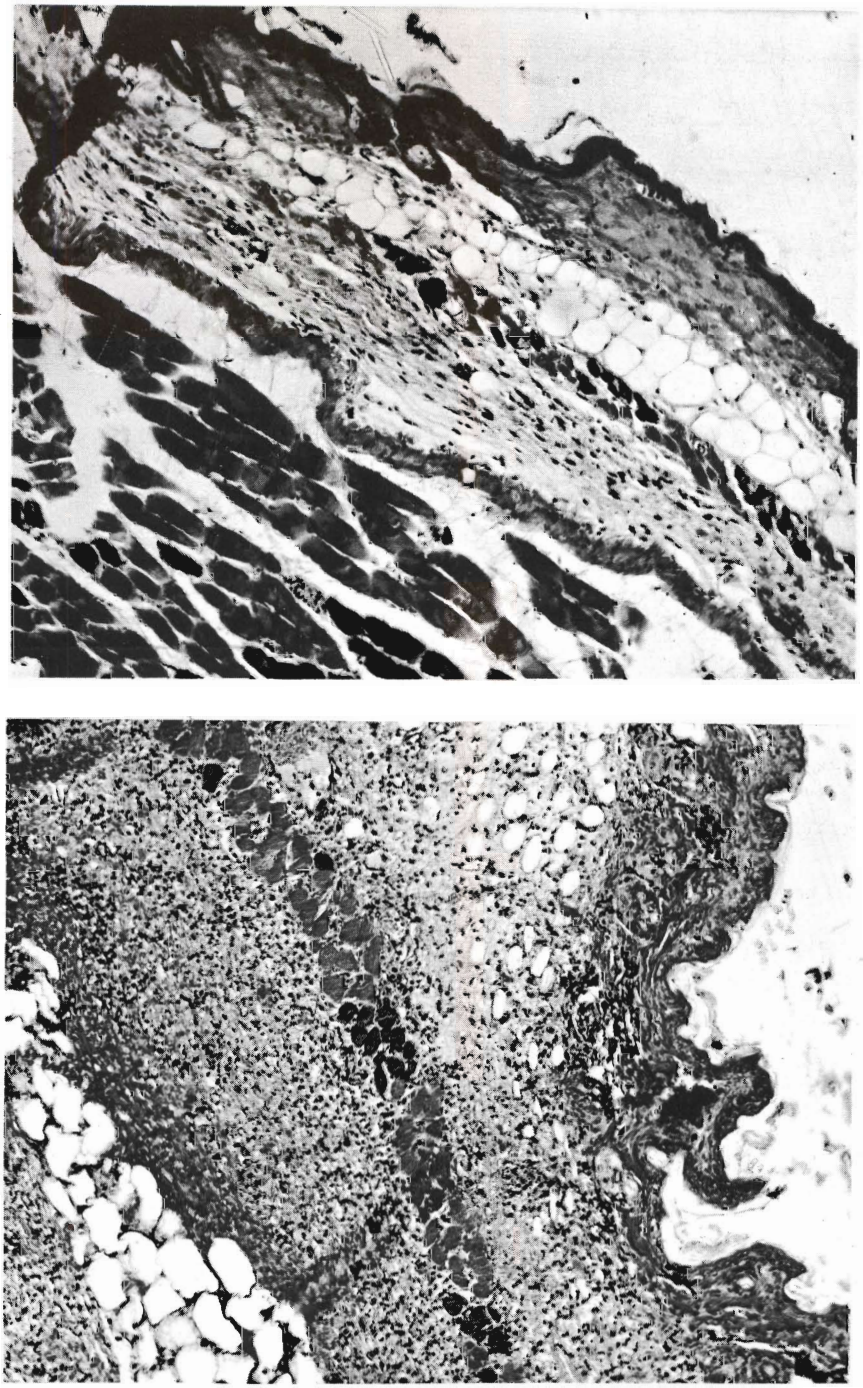

Fig. 4. Cutaneous reaction of grasshopper mice, Onychomys leucogaster, following intradermal injection of a heat-killed suspension of Pasteurella tularensis, strain Schu A. (Upper) Control grasshopper mouse. (Lower) Past. tularensis challenged deer mice. Observations 24 hours after skin testing. Bouin's fixation; haematoxylin and eosin. X100.

G. Characterization of Tularemia Hypersensitivity in Guinea Pigs.

1. Development of Hypersensitivity in Guinea Pigs after Subcutaneous Inoculation with 10^9 Living Pasteurella tularensis Jap_4 Cells.

This part of the study was carried out to observe the development of hypersensitivity following challenge with living Past. tularensis strain Jap_4 cells into guinea pigs. Forty guinea pigs inoculated with Past. tularensis strain Jap_4 were divided into eight groups of five pigs each. At intervals of 0, 3, 4, 5, 7, 8, 10 and 12 days following inoculation a different group of 5 guinea pigs each was skin tested. One tenth of a ml of a heat killed suspension of Past. tularensis strain Schu A was injected on one side of the abdominal area and Past. tularensis polysaccharide on the other side. Each guinea pig was skin tested but once. The injection sites were observed at 0.5, 2, 4, 6, 24 and 48 hours following intradermal injection of the whole cell antigen and polysaccharide.

Results of the observations on the guinea pigs after injection of the heat killed whole cell antigen are presented in Table 12. At the 4 and 24 hour observation times none of the five guinea pigs tested on the 0, 3rd, or 4th day reacted. Skin reactivity was apparent in two of the five guinea pigs tested on the 5th day, and was more apparent in the five guinea pigs tested with the whole cell antigen on the 7th and 8th days following challenge with Jap_4. The five tested on the 10th day, for reasons unknown, failed to react to intradermal injection of the whole cell antigen. Two and possibly three of the five guinea pigs tested on the 12th day demonstrated cutaneous reactivity. The three guinea pigs that showed positive reactivity 4 hours after skin test 12 days after challenge elicited stronger reactions when observed at 24 hours. A fourth animal, that was apparently unreactive at 4 hours (6 mm x 6 mm), exhibited a reaction measuring 12 mm x 12 mm at 24 hours.

TABLE 12

Development of cutaneous sensitivity[1] to intradermal injection of heat killed Schu A cell suspension into guinea pigs following challenge with 10^9 *Pasteurella tularensis* strain Jap_4 cells.

Animal number	Time in days following inoculation of Jap_4							
	0	3	4	5	7	8	10	12
	4-hours after intradermal injection of antigen							
1	6	7	4	7	12	20	5	9
2	6	6	5	9	15	18	6	6
3	5	7	4	11	13	21	7	10
4	6	7	4	8	10	14	6	6
5	6	7	0	13	14	13	7	16
	24-hours after intradermal injection of antigen							
1	6	6	3	7	8	20	9	16
2	6	6	5	4	8	8	7	8
3	4	5	6	6	7	11	5	20
4	5	6	0	5	6	14	6	12
5	6	7	0	3	9	8	5	14

[1]Expressed as the diameter of the reaction in mm.

Results of the observations of the guinea pigs skin tested with Past. tularensis polysaccharide are presented in Table 13. None of the guinea pigs tested through the third day reacted to the polysaccharide. On the fourth day, one and possibly three guinea pigs developed slight reactions 4 hours after intradermal injection of polysaccharide, but these diminished in 24 hours. Of the remaining guinea pigs, each group of 5 tested at 7, 8, 10 and 12 days developed moderate to strong skin reactions observable after 4 hours from the time of the injection of the skin test dose. Unless the skin reaction was strong at 4 hours, the reaction site had markedly decreased in size when observed 20 hours later. The strongly reactive sites of most tested animals was noticeably decreased but still evident when observed at 24 hours.

2. Quantitative Study of the Arthus Type of Hypersensitivity Passively Induced in the Guinea Pig and Rabbit.

This part of the study of Past. tularensis hypersensitivity was conducted to investigate the level of circulating Past. tularensis antibody that was required to cause a skin test reaction to 90 mg of polysaccharide.

Serum samples were collected aseptically from guinea pigs three weeks after having been challenged with 10^9 Jap_4 cells. All serum samples were stored at -20 C until used. Each was analyzed for Past. tularensis polysaccharide antibody nitrogen as previously described.

One tenth of a ml of each serum sample was injected intradermally at four different sites on the shaven back of a normal healthy guinea pig. Twenty-four hours later two of the sites were tested by intradermal injection of the heat killed whole cell antigen and two were tested by intradermal injection of the Past. tularensis polysaccharide. Thus, each serum sample was tested in duplicate for passive transfer of cutaneous sensitivity.

TABLE 13

Development of cutaneous sensitivity[1] to intradermal injection of polysaccharide into guinea pigs following challenge with 10^9 Pasteurella tularensis strain Jap_4 cells.

Animal number	Time in days following inoculation of Jap_4							
	0	3	4	5	7	8	10	12
	4-hours after intradermal injection of antigen							
1	5	6	12	10	13	20	20	15
2	8	6	10	12	16	18	19	16
3	5	5	10	13	16	21	14	15
4	7	6	7	11	14	14	19	20
5	8	7	0	8	13	13	23	18
	24-hours after intradermal injection of antigen							
1	7	5	4	6	7	8	7	8
2	6	5	8	6	10	10	9	4
3	6	5	9	12	12	9	6	14
4	4	5	0	6	11	7	10	9
5	5	5	0	6	10	6	10	10

[1]Expressed as the diameter of the reaction in mm.

Only non-pregnant female guinea pigs weighing 300-350 grams were used.

Passive transfer studies of the Arthus type of reaction, using guinea pigs and rabbits as the recipient animals of anti-Past. tularensis serum are tabulated in Tables 14 and 15.

Guinea pig serum containing 0.027 mg of Past. tularensis antibody nitrogen failed to produce local sensitivity when transferred to a normal guinea pig. Pasteurella tularensis antibody nitrogen in concentrations in excess of 0.176 mg successfully transferred sensitivity to normal animals. From data in Table 14 it appears that concentrations above 0.176 mg antibody nitrogen transferred to normal guinea pigs increases but slightly the severity of subsequent skin tests.

The local reaction following skin test of the sensitized site of the recipient animal reached a maximal response four to six hours after injection of test antigens. After this period the reactions gradually subsided until at 24 hours, all but the severest reactions had been greatly reduced.

Based on observations at 24 hours, normal rabbits proved to be as susceptible, if not more so, to local sensitization with anti-Past. tularensis serum as did guinea pigs. Sensitized and normal rabbits responded to both skin test preparations by developing a non-specific cutaneous reaction measuring 51 mm x 15 mm, 4 hours after skin test. This non-specific response was sufficient to mask any specific reactions which may have occurred during the first four hours following injection of the skin test antigen. When the sensitized rabbits were observed 24 hours following intradermal injection of the antigen, there was a striking specific response to both skin test preparations at the sites of local sensitization. Antibody concentrations as low as 0.055 mg nitrogen were sufficient to sensitize

TABLE 14

Serum passive transfer of the Arthus type hypersensitivity in the guinea pig with Pasteurella tularensis antiserum.

Guinea pig Serum No.	Mg Antibody N	Skin reaction to Heat killed Schu A antigen[1]		Skin reaction to Past. tularensis polysaccharide[1]	
		4 hours	24 hours	4 hours	24 hours
1	0.027	6	5	7	8
2	0.176	5	7	13	6
3	0.271	11	6	20	7
4	0.320	11	6	13	6
5	0.426	8	7	15	10
6	0.451	12	12	22	16
7	0.490	16	15	17	16
8	0.521	12	11	19	10
9	0.533	12	9	13	8
10	0.556	11	5	17	9
11	0.613	12	5	14	5
12	0.688	9	5	14	8
13	0.815	15	12	19	6
14	0.900	9	7	14	7
15	1.231	12	7	16	8
Control	0.0	7	5	5	6
Control	0.0	6	5	7	7
Control	0.0	7	7	6	4

[1]Expressed as the diameter of the reaction in mm.

TABLE 15

Serum passive transfer of the Arthus type of hypersensitivity by transferring guinea pig anti-*Pasteurella tularensis* serum to rabbits.

Guinea pig Serum No.	Mg Antibody N	Skin reaction to Heat killed Schu A antigen[1]		Skin reaction to *Past. tularensis* polysaccharide[1]	
		4 hours	24 hours	4 hours	24 hours
1	0.220	15	10	15	17
2	0.110	15	10	15	13
3	0.055	15	6	15	11
4	0.207	15	3	15	0
5	0.013	15	3	15	3
Control	0.0	15	3	15	3

[1]Expressed as the diameter of the reaction in mm.

local areas of the rabbit to subsequent intradermal injection of both preparations.

3. Cellular Transfer of Tularemia Hypersensitivity after Sensitization with Pasteurella tularensis Jap_4.

Thirty-eight guinea pigs each given a single cutaneous inoculation of 10^9 living Past. tularensis Jap_4 cells were used to prepare peritoneal exudates for detection of the presence of delayed hypersensitivity by cellular transfer of activity. In order to determine the percentage of viability that was maintained in the exudate cells when handled by the described method, the neutral red vital staining technique was utilized (Cowdry, 1952). Viability counts were made on two separate exudate pools. In the first exudate pool, 300 exudate cells were counted on two slides and 75 per cent were classified as viable. In the second exudate pool, 500 cells were counted, one hundred on each of five slides. Seventy-eight per cent of the 500 cells counted were classified as viable.

One week following inoculation of the organisms, peritoneal exudates from one group of 4 guinea pigs and one group of 6 guinea pigs were collected and pooled. A volume of 0.95 ml of packed cells obtained from the pool of four exudates and a volume of 1.25 ml of packed cells from the pool of six exudates. The cells were immediately transferred as previously described to recipient guinea pigs. Twenty four hours after transfer of the exudate cells, two recipient animals were skin tested with both the heat killed whole cell antigen and the polysaccharide. No reactions above that also observed in control guinea pigs were observed.

Two weeks after injection of the sensitizing organisms, peritoneal exudates were collected from 15 guinea pigs. The exudates were grouped into three pools of the exudates of five guinea pigs each. A packed

exudate cell volume of 0.5, 0.5, and 1.7 ml were obtained from the three respective pools.

The two guinea pigs having received the 0.5 ml of packed cells failed to respond to the intradermal injection of the heat killed Schu A suspension. However, the recipient guinea pig receiving the 1.7 ml volume of packed exudate cells developed an erythematous area 12 mm in diameter with central vesiculation.

Three weeks after injection of the sensitizing organisms, peritoneal exudates were collected from eight more guinea pigs in two pools of four exudates each. One exudate contained 0.2 ml of packed cells and the second 0.6 ml of packed cells. The same procedure for transfer of exudate cells and skin testing of the recipient animals was followed. The normal guinea pig which received 0.2 ml of packed cells developed a slight erythema measuring 5 mm in diameter 24 hours after skin test with the heat killed Schu A suspension. The guinea pig having received 0.6 ml of packed cells developed an erythema measuring 12 mm in diameter, 24 hours after skin testing with the heat killed Schu A suspension.

An attempt to transfer sensitivity at four weeks following injection of Jap_4 organisms, using an exudate pool of five guinea pigs that yielded 0.8 ml packed exudate cells was also successful. Twenty-four hours after skin test with the heat killed Schu A cell suspension, the recipient guinea pig had developed an erythematous zone 13 mm in diameter, with central necrosis. These same recipient guinea pigs were also skin tested with the polysaccharide at the time they were skin tested with the heat killed Schu A suspension. None of the recipient guinea pigs developed a positive reaction to the polysaccharide.

In all experiments normal controls not having received exudate cells were skin tested in the same manner as were the recipient animals.

The usual response of the control guinea pigs to the intradermal injection of the heat killed suspension was an indurated area measuring 3 mm to 5 mm in diameter. Intradermal injection of polysaccharide resulted in a slight erythema of 3 mm to 7 mm in diameter. When skin lesions were observed 48 hours after injection of the antigen, it was found that the positive reactors had only a slight decrease in the size of the skin reaction while the cutaneous response of the control animals and negative recipient guinea pigs had decreased markedly and in many cases was not detectable.

H. A Study of the Persistence of Tularemia Complement-Fixing and Agglutinating Antibodies in Pasteurella tularensis Challenged Rodents.

The antibody response of 100 deer mice, 21 Ord kangaroo rats, and 24 desert wood rats as a result of challenge with 10^3 Past. tularensis strain Jap_4 is summarized in Table 16.

The serological response of deer mice was based on analysis of seven pools of sera each representing 10 mice, whereas the results reported for the Ord kangaroo rats and wood rats are based on titers of individual serum samples. The complement fixing and agglutinating titers of deer mice reached a peak of 1:1280 and 1:320 respectively three weeks after challenge. Both types of antibody titers decreased at about the same rate until both were 1:40 at 25 weeks. The agglutinin titer became negative by the 31st week while the complement fixing titer remained at 1:40. By the 48th week the complement fixing titer was also negative.

The complement fixing and agglutinin titers of Ord kangaroo rats varied markedly from those of the deer mice. There was no complement fixing titer over 1:64 at any time following infection. Complement fixing antibodies could not be detected 3 weeks after challenge, but were

TABLE 16

The persistence of agglutinin and complement fixing antibodies in deer mice, Ord kangaroo rats and desert wood rats following challenge with 10^3 *Pasteurella tularensis* Jap_4 cells.

Time in weeks following challenge	Deer mice[1]		Ord kangaroo rats		Desert wood rats	
	Agglutinin titer[2]	Complement fixing titer[3]	Agglutinin titer	Complement fixing titer	Agglutinin titer	Complement fixing titer
1	40					
2	160	640				
3	320	1280	0(1) 320(1) 640(2)	0(4)		
4					160(1) 640(3)	40(1) 80(2) 160(3)
7	160	160	0(2)	16(1) 32(2) 64(2)	20(1) 160(3)	24(2) 40(2)
11					40(1) 80(2) 160(2)	20(2) 40(1) 80(2)
12		80				
18	160	20	320(1) 640(2) 1280(1)	0(2) 16(1) 64(1)	0(1) 40(2) 80(2)	0(2) 16(2) 32(1)
25	40	40	20(1) 80(2) 320(1)	0(4)	0(3) 20(1)	0(2) 16(1) 32(1)
31	0	40				
40	0	0	160(2) 320(1) 640(1)	0(3) 32(1)		
48	0	0				
53	0	0				

[1]Each deer mouse titer represents a pool of 10 serum samples.
[2]Titers shown as the reciprocal of the highest serum dilution agglutinating Past. *tularensis* antigen to +2 or greater.
[3]Titers shown as the reciprocal of the highest serum dilution showing 50% lysis of sensitized sheep red blood cells.
()Numbers in parentheses indicate number of serum samples with indicated titer.

detected by the 7th week. The agglutinin antibody response was comparatively higher. Agglutinin titers of 1:320 or greater were detected in individuals at each time period of sampling up through the last sampling at 40 weeks. Both types of antibody produced by the Ord kangaroo rats did not demonstrate any marked peak titer, but maintained a fairly constant level over the 40 week period.

The desert rat produced an antibody response similar to that observed in the deer mouse. Peak titers of 1:160 to 1:640 were reached 3 weeks following challenge and gradually decreased to titers of 1:32 or below by 25 weeks.

V. DISCUSSION

A characteristic cutaneous reaction on following intradermal injection of Past. tularensis, heat killed Schu A cells, has been observed in separate groups of five species of wild rodents previously challenged with Past. tularensis organisms of attenuated virulence. The characteristic reaction observed here was unlike that previously reported by Tumanskii and Kolesnikova (1939) with species of Citellus, Lagurus, Cricetus, Alactaga, and Microtus. These authors trapped 123 rodents of these species in an area where there was an active tularemia epizootic in the rodent population. Eleven positive reactors were observed following intracutaneous injection of 0.1 and 0.2 ml containing 25 to 50 million heat killed Past. tularensis organisms. A positive reaction was described as erythema and edema leading ultimately to ulceration when observed from the under side of the skin. The difference in the reaction of the rodents reported here and that reported by Tumanskii and Kolensnikova (1939) may have been due to variation in the response of different species, antigen preparation, method of injection of the antigen and/or volume of antigen suspension.

In the study of the serological response of various rodent species following challenge with Past. tularensis, some marked variations between species were found. Deer mice and wood rats were observed to develop an agglutinin response of similar magnitude and persistence. The Ord kangaroo rat, however, developed one or two-fold greater agglutinin titers and maintained these high titers for periods of at least 40 weeks. The agglutinin titer of the deer mouse was negative at 40 weeks, and the agglutinin titer of the wood rat was 1:20 or below at 25 weeks.

All rodent species produced complement fixing antibody titers of similar levels except deer mice which produced slightly higher titers the

first 7 weeks following challenge. Low level complement fixing antibody could be detected in deer mice, Ord kangaroo rats, and wood rats 31, 40, and 25 weeks respectively, following challenge.

It is evident that in the serological tests for Past. tularensis antibodies in these species, complement fixation procedures would add little to information obtained from the use of tube agglutination methods. The use of the skin test in supplementing the agglutination test would possibly be of value in that the test animals remain alive to be used for further studies.

The relation of serological response to the expected life span of these rodents is of particular interest. The approximate life span of the deer mouse in the field is 36 to 40 weeks; of the wood rat 72-80 weeks; and of the kangaroo rat 64-72 (Vest, 1959). Assuming tularemia infection occurred while the rodents were young, it is interesting to compare the expected life spans to the persistence of tularemia antibodies and cutaneous sensitivity. It appears that the complement fixation test and the skin test could detect past Past. tularensis infection in the deer mouse throughout the expected life span of this animal. The agglutinin antibody in the deer mouse may become undetectable during this period. In Ord kangaroo rats which have recovered from Past. tularensis infection the complement fixation test appears to be of little value, while the skin test is adequate to detect past infection for most animals tested through half the rodent's expected life span. The agglutination test is apparently superior to the other tests in detecting past Past. tularensis infections in this species. In the wood rat, retrospective diagnosis of tularemia by complement fixation and agglutination tests infection would be difficult beyond a third to one-half the rodent's expected life span. All skin tests of Past. tularensis

recovered wood rats were positive through a period approximating one third of the animal's expected life span.

The results of the study of 181 rodents trapped in an area where there was a known tularemia outbreak in the rabbits must be interpreted with care. It may be stated that the rodents tested were probably not involved in the tularemia outbreak. Whether or not, in general, rodents were involved would still be of question. It has been suspected that rodents sick of tularemia would remain in their burrows until death. Stoenner, (1958) has indicated that tularemia is uniformly fatal for species of rodents that develop natural infections, although this point has been questioned by Marchette et al. (1961). Laboratory observations on the virulence of the two strains of Past. tularensis isolated from the cottontail and the jack rabbit carcasses demonstrated that they did produce uniformly fatal infections in deer mice and wood rats (Marchette et al. 1961). It may then be assumed that if this outbreak involved rodents they may have died in their burrows and therefore were not trapped.

Cutaneous hypersensitivity to Past. tularensis polysaccharide developed to a mild degree in guinea pigs four days following challenge with Past. tularensis, strain Jap4 (Tables 14 and 15). This reactivity increased with time until peak reactions were observed on the 8th, 10th, and 12th days following challenge. In contrast, cutaneous sensitivity to the whole cell antigen was not detected until the 5th day, and increased to a maximal level by the 8th day. The non-reactivity of some animals at the 10th and 12th days cannot be explained.

The latent period of 4 to 5 days before the development of cutaneous hypersensitivity was in general in agreement with results reported by Kalitina (1958). He reported that skin test reactivity occurred on the 6th day following the vaccination of guinea pigs with the NIIEG strain of

Past. tularensis. The development of cutaneous sensitivity in humans has been reported to occur on or before the 8th day following infection (Foshay, 1934).

It is apparent from examining Tables 14 and 15 that the intradermal injection of anti-Past. tularensis serum and subsequent skin testing of the site with polysaccharide may be used to detect the presence of Past. tularensis antibody. Antibody nitrogen concentration of 0.055 mg could be detected by this method in the rabbit and 0.176 mg or greater using the guinea pig as the recipient animal. This method could be used to supplement studies on the development of the Arthus type of reaction following challenge of guinea pigs with sublethal doses of Past. tularensis.

It is evident from the studies of the cellular transfer of cutaneous sensitivity that delayed type hypersensitivity had not reached a detectable level during the first week following challenge. This was indicated by the inability to transfer cutaneous hypersensitivity with peritoneal exudate cells during the first week following challenge with Past. tularensis.

When cellular transfer of tularemia sensitivity was accomplished, recipient guinea pigs reacted to intradermal injection of the heat killed Schu A antigen but not to intradermal injection of the polysaccharide. This would suggest that the whole cell antigen contained a substance not present in the polysaccharide preparation that would elicit the delayed type of hypersensitivity response.

Observations of the development of cutaneous reactions following intradermal injection of the Past. tularensis polysaccharide of heat killed Schu A antigen into Past. tularensis challenged guinea pigs indicated that this response was similar to the Arthus type of reaction. The similarity of the cutaneous response to the intradermal injection of the polysaccharide to that of the Arthus type of reaction was supported by the transfer of this

reaction to normal animals with serum from sensitized animals. Cutaneous sensitivity to intradermal injection of the polysaccharide was not transferred by peritoneal exudate cells of the sensitive animals. The cellular and serum transfer of cutaneous sensitivity indicates that the response elicited by intradermal injection of the heat killed Schu A antigen was a combination of the delayed type of hypersensitivity and the Arthus type of reaction.

VI. SUMMARY

Five species of wild rodents native to the Great Salt Lake Basin have been found to produce a characteristic cutaneous response to intradermal injection of various Past. tularensis preparations, following challenge with viable Past. tularensis organisms. An area of induration was produced which reached maximal size at 48 hours following intradermal injection of the Past. tularensis preparations. Histologically this reaction appeared to be a typical acute inflammatory process. Cutaneous sensitivity as a result of intradermal injection of heat killed Past. tularensis cells into challenged deer mice, Ord kangaroo rats, and desert wood rats persisted for up to 53, 40 and 25 weeks respectively.

Deer mice challenged with Br. suis did not react to intradermal injection of heat killed Schu A strain cells whereas reactors were observed among deer mice challenged with Br. neotomae.

White mice, guinea pigs and albino rabbits challenged with Past. tularensis were also observed to develop characteristic responses following intradermal injection of various Past. tularensis preparations. The cutaneous response of the challenged white mice was similar to that of the challenged wild rodents. In challenged guinea pigs the cutaneous response was one of edema and erythema which was maximal 24 hours after skin test. The response of rabbits to intradermal injection of Past. tularensis preparations was an erythematous area attaining a maximum size 48 hours after skin test.

The occurrence and development of cutaneous sensitivity in guinea pigs following challenge with Past. tularensis, strain Jap_4, was studied. It was observed that cutaneous sensitivity may develop 4 to 5 days following challenge. Passive transfer studies with peritoneal exudate cells indicated a lack of detectable delayed hypersensitivity during the first week

following challenge. However, delayed hypersensitivity was demonstrated in guinea pigs two, three and four weeks following challenge with the Jap_4 strain of Past. tularensis.

A method of quantitative determination of Past. tularensis antibody precipitated by polysaccharide was established and used to determine the antibody nitrogen necessary to evoke local cutaneous sensitivity to Past. tularensis skin test antigens in normal rabbits and guinea pigs.

Experimental evidence demonstrated that the cutaneous response of Past. tularensis challenged guinea pigs to intradermal injection of polysaccharide was similar to the Arthus type of reaction. It was also demonstrated that the cutaneous response of challenged guinea pigs to intradermal injection of heat killed Past. tularensis Schu A organisms was a combination of the Arthus type and the delayed type of hypersensitivity response.

The persistence of Past. tularensis complement fixing and agglutinating antibodies in Past. tularensis Jap_4 challenged deer mice, Ord kangaroo rats and wood rats was studied. In deer mice complement fixing antibody persisted for 31 weeks and agglutinating antibody for 25 weeks. Antibody detected by both tests persisted in Ord kangaroo rats for at least 40 weeks, although the agglutinin titers were much higher than the complement fixing titers. In challenged wood rats, both tests were positive in some animals 25 weeks following challenge.

The relative values of the skin tests, agglutination test, and complement fixation test to detect Past. tularensis infection in rodents were discussed.

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