STUDIES ON SCRUB TYPHUS (TSUTSUGAMUSHI DISEASE)

III. Heterogenicity of Strains of R. tsutsugamushi as Demonstrated by Cross-Vaccination Studies*

By FRED L. RIGHTS, Ph.D., and JOSEPH E. SMADEL, M.D.,
Assisted by ELIZABETH B. JACKSON

(From the Department of Virus and Rickettsial Diseases, Army Medical Department Research and Graduate School, Washington, D. C., and the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, January 6, 1948)

Although animals which recover from disease induced by one strain of *Rickettsia tsutsugamushi* are resistant to infection with heterologous strains (1–10), all organisms now grouped in this subgenus are not antigenically homogeneous. Evidence for heterogeneity is obtained in serological studies which employ complement fixation (11, 12), serum protection (13), and toxin-antitoxin techniques (14). Non-infectious vaccines of several types are capable of immunizing mice against the homologous strain of *R. tsutsugamushi* (15–17) but the degree of protection elicited by such materials against heterologous strains is unknown. The present report supplies information on this point and provides additional evidence of antigenic differences among the members of the scrub typhus group of agents.

Materials and Methods

Strains of R. tsutsugamushi.—The eight strains of *R. tsutsugamushi* employed in the current investigation were selected to represent the agent as obtained from various geographical areas and from different zoological sources; *i.e.*, men, mites, and rats. The origin and lineal descent of these strains are given in some detail in the following paragraphs since it appears desirable to establish these points clearly. Certain of the immunological and serological data which indicate the known relationships of the eight to each other, or to additional strains of *R. tsutsugamushi*, are summarized in Table I; this information has been presented in detail elsewhere (18). The toxin-antitoxin reaction is not mentioned in Table I because only the Gilliam strain has yielded a toxin and none of the antisera against the reference strains contains appreciable amounts of specific antitoxin (14).

The Imphal 8 strain was recovered late in 1943 from a pool of blood clots obtained from five patients with tsutsugamushi disease who were hospitalized in the Imphal Valley on the Indian-Burmese border. The characteristics of this strain shortly after isolation have been reported (19). In the present work, seed inoculum for the preparation of vaccine and material for challenging vaccinated mice were obtained from yolk sacs of the 47th to 53rd passages. Since the original isolation from human beings, this strain had been through one guinea pig, eight rabbits, one mouse, and the designated number of egg passages.

The Karp strain was originally obtained by Dr. E. Derrick of Brisbane, Australia, from

*Submitted by Fred Lewis Rights in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Faculty of Pure Science, Columbia University, May 16, 1947.

blood taken in January, 1943, from a soldier who contracted the disease in the Buna-Guna area of New Guinea. The Karp agent was used previously in this laboratory for the prepara-

TABLE I

Antigenic Relationships of Strains of R. tsutsugamushi

Reference strains	Immune animals resist challenge with (strain)		Serological tests with antisera prepared against reference strains					
			Serum neutralization			Complement fixation		
			Challenge strain		Result*	Antigen		Result*
Imphal 8	Calcutta	(10‡)				Calcutta	(19)	++
Karp	Gilliam	(8)						
	Seerangayee	(8)						
	Buie (case 9)	(8)				Gilliam	(11)	+−
	Ceylon	(9)				Seerangayee	(11)	+−
	Calcutta	(9)				Buie (case 9)	(11)	+
	Imphal 8 or 13	(9)						
Kostival	Mite 21	(6)	Mite 21	(13)	+−			
	Mite 22	(6)	Seerangayee	(13)	+−			
Mite 21	Kostival	(6)	Kostival	(13)	+−			
			Seerangayee	(13)	+−			
Wild rat 235	135 Wild rat	(7)						
	370 Mite	(7)						
	Shope	(7)						
Pescadores	Niigata	(3)						
Seerangayee	6 Malayan strains	(2)						
	2 Sumatran strains	(2)	Kostival	(13)	+−	Gilliam	(11)	+−
	Karp	(8)	Mite 21	(13)	+−	Karp	(11)	+−
	Gilliam	(8)						
	Buie (case 9)	(8)						
	Volner	(10)						
Volner	Seerangayee	(10)						

* +− indicates a slightly positive or questionable result.
+ indicates a positive reaction and a partial or close relationship.
++ indicates a positive reaction equivalent to that obtained with the homologous material; by this method the agents are indistinguishable.
‡ Numbers indicate references cited in Bibliography.

tion of vaccine by two different methods (16, 17). Suspensions of tissue of the 27th to 41st yolk sac passages were used in the current studies.

The Kostival strain was recovered in November, 1943, from a patient in the Dobadura area of New Guinea by Blake and his coworkers (6). It also has been employed in earlier studies of scrub typhus vaccine in this laboratory (17). The 101st to 128th yolk sac passages of the Kostival strain provided material for the present work.

The mite 21 strain (also referred to as host 21) (13, 14) was isolated in mice during Novem-

ber, 1943, by Blake and his associates from a pool of mites (*T. fletcheri*) which were obtained from a bandicoot caught in the Dobadura area, New Guinea. Blood from a mouse of the sixth passage was injected into embryonated eggs (6) and yolk sac material from the 6th to 24th subcultures of this line was employed in the current work.

The wild rat 235 strain (also referred to as wild rat 2 (14)), was obtained during April, 1944, by Kohls and his coworkers (7) from the pooled brains of four wild rats (*Rattus concolor browni*) which were trapped in the Dobadura area of New Guinea. A pool of liver and spleen tissue from mice of the fourth passage served as challenge material throughout the present experiments.

The Pescadores strain was originally isolated in 1935 (3) from a native of the Pescadores Islands, and has been extensively employed in Japan (3, 20). This agent, which was temporarily lost by the Japanese, was recovered by them in December, 1945, from an axillary lymph node of a paretic who had been infected for therapeutic purposes with the strain 5 months previously (21). This reisolation was accomplished in a Mongolian hamster (*Meriones unquiculatus* M. Edward) and the agent subsequently was passed to mice wherein it showed all the usual characteristics of a strain of *R. tsutsugamushi*.[1] Portions of a suspension of pooled liver and spleen tissues from mice of the sixth passage made in this laboratory were used as challenge material throughout the present work.

The Seerangayee strain was recovered from a patient with scrub typhus in Malaya in 1934 (22). This agent was taken to Australia by Dr. R. Lewthwaite several weeks before the fall of Singapore in 1942. It had been carefully compared with other Malayan and Sumatran strains before World War II and therefore assumes considerable importance in relating pre- and postwar phases of scrub typhus investigation. The characteristics of recent passages of this strain in various hosts have been redescribed in some detail (8). The agent was brought to this country as infected yolk sac tissue in February, 1944, and was received at the Army Medical Department Research and Graduate School, from the National Institute of Health as infected guinea pig tissue. Subsequent passages were made in mice, and frozen suspensions of pooled liver and spleen tissue from mice of the second and third passages at the School provided challenge material for the present studies.

The Volner strain (also referred to as J.H.V. (23)) was recovered in 1945 in mice inoculated with blood from a patient on the island of Samar in the Philippines (23). Suspensions of pooled liver and spleen tissue from mice of the sixth and seventh passages were employed as challenge material in the current experiments.

Laboratory Animals.—Swiss mice of the Bagg Farm stock weighing 18 to 21 gm. were employed for all titrations and vaccination experiments. White rats which weighed 125 to 225 gm. were used to furnish infected tissues for the preparation of vaccines. Six or seven day old embryonated eggs were employed for the maintenance of strains and the preparation of highly infectious seed inocula for the rats. These eggs were inoculated into the yolk sac and were subsequently maintained at a temperature of 35°C. until harvested; *i.e.*, when the embryos were moribund 7 to 9 days later.

Preparation of Vaccines.—Vaccines were prepared from infected white rat tissues as previously described (16, 19); a résumé of this method follows. Freshly prepared suspensions of yolk sac tissue rich in rickettsiae were cleared of coarse particles and large fat droplets by light centrifugation; 2.0 cc. amounts were then injected intravenously into white rats. When the majority of the 20 animals inoculated with a given seed material were moribund, *i.e.* some-

[1] *R. tsutsugamushi* has been recovered from the brains of white rats from 14 to 98 days after initial infection (7) and Major B. L. Bennett, Sanitary Corps, of this laboratory has reisolated scrub typhus organisms from the spleens of mice which had been infected 10 months previously.

time during the 4th to 6th days, the lungs and spleens of recently dead and of sick rats were removed with sterile precautions, pooled, weighed, and then ground in a mortar with a small quantity of alundum. A 10 per cent suspension of the infected tissue was prepared from this triturated material using a solution consisting of 98 parts of 0.9 per cent NaCl solution and two parts of McIlvane's buffer (pH 7.4). The resultant suspension was cleared of coarse particles by sedimentation at 1,500 R.P.M. for 5 minutes in a horizontal centrifuge and the supernatant fluid was withdrawn and saved. Immediately after samples had been removed for infectivity tests, sufficient U.S.P. formaldehyde solution and merthiolate were added to the suspension to bring their final concentrations to 0.1 and 0.01 per cent, respectively. Such tissue suspensions, after storage for several weeks at 5°C. to permit inactivation of rickettsiae, constituted the vaccines; none were infectious when employed in immunity studies. Frequently several batches of vaccine, which were prepared from a single strain of scrub typhus rickettsiae, were pooled.

Vaccines from four strains of *R. tsutsugamushi* were prepared by this method. A number of these failed to meet the criteria previously established (16) for a potent antigen and were not employed in the current work.

Determination of Infective Titers.—Serial tenfold dilutions of the suspensions of infected tissues were prepared in skimmed milk medium (Difco, pH 7.2) and each mouse in a group of five to ten animals was inoculated intraperitoneally with 0.2 cc. of one of the test dilutions. The animals were observed for 21 days and deaths recorded. All mice which succumbed earlier or later than the expected time of death were autopsied; if they had no pathological evidence of experimental scrub typhus infection they were dropped from the group. In addition, the cause of death was determined at autopsy in representative animals which succumbed following typical disease in the groups which received dilutions of rickettsiae in and near the end point range. From the accumulated deaths and survivals of the test animals the 50 per cent lethal end points were calculated by the method of Reed and Muench (24).

Immunogenic Assay of Vaccines.—The capacity of a given vaccine to induce resistance to the homologous and heterologous strains of *R. tsutsugamushi* was determined in the following manner. Batches of 150 to 500 mice were segregated according to sex in groups of eight or ten per jar and each mouse was given an intraperitoneal injection of 0.5 cc. of vaccine on three occasions at intervals of 5 days. Two weeks after the final injection of vaccine, groups of eight or ten mice were challenged by the intraperitoneal injection of 0.2 cc. amounts of an appropriate dilution of a previously standardized suspension of infectious material which had been stored at −70°C. Following challenge inoculation immunized animals, as well as control mice of approximately the same weight which received similar infectious material, were observed for 3 weeks and deaths were recorded. The infectious titers of the rickettsial material in control and test mice were calculated as described above. The degree of immunity induced by a given vaccine against a strain of *R. tsutsugamushi* was expressed as an immunity index. This represented the algebraic difference between the logarithms of the infectious titers determined in control and vaccinated mice which had received comparable challenge material.

EXPERIMENTAL

Scrub typhus vaccines prepared from the four selected strains of *R. tsutsugamushi*, which appeared suitable for the purpose, were tested for their capacity to immunize mice against infections with a number of strains of the agent. The latter were chosen as representative of the species as recovered from different hosts in various geographic areas. The results of these experiments provide data which bear directly on studies dealing with the immunization of man against this disease.

Resistance of Mice Immunized with Scrub Typhus Vaccines to Infection with Eight Strains of R. tsutsugamushi.—Groups of mice were immunized with vaccine prepared from tissues of rats infected with the Imphal strain and were subsequently challenged intraperitoneally with suspensions of infectious material containing 1 to 100,000 or 1,000,000 lethal doses (LD) of *R. tsutsugamushi* of the Imphal strain or of one of seven other strains of scrub typhus. At this time normal mice of the same weight as the vaccinated animals were injected with portions of each challenge suspension. Results illustrative of those obtained in a typical experiment are summarized in Table II. The data are pre-

TABLE II

Resistance of Mice Immunized with Imphal Vaccine to Challenge Infection with Strains of R. tsutsugamushi

Rickettsial strain	Vaccinated mice*	Challenge inoculation: Dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Infective titer 50 per cent (lethal)	Immunity index‡
Imphal 8	Controls		10/10	10/10	9/10	10/10	9/10	4/10	1/10	0/10§		$10^{-6.8}$	
	Vaccinated	7/8	8/10	8/10	4/10	1/10	0/10	0/10	0/9			$10^{-9.7}$	3.1
Imphal 8	Controls		10/10	10/10	10/10	9/10	3/9	7/9	2/10	0/10		$10^{-7.0}$	
	Vaccinated		7/8	5/10	5/10	1/9	2/8	1/10	0/8			$10^{-9.8}$	3.2
Karp	Controls				10/10	10/10	10/10	10/10	4/10	3/10	0/10	$10^{-8.1}$	
	Vaccinated			10/10	10/10	8/10	8/10	4/10	5/10	0/9		$10^{-6.9}$	1.2
Seerangayee	Controls				10/10	9/9	9/10	4/10	1/9	0/10		$10^{-6.8}$	
	Vaccinated				10/10	8/10	9/10	3/10	1/10			$10^{-6.6}$	0.2

* Mice were immunized with vaccine 1-2-5 prepared from white rats infected with Imphal 8 strain; a summary of this data is included in Table III.

‡ Immunity index represents the difference between exponential values of the infective titers in the contro and test groups.

§ Numerator indicates the number of mice in each group that died of the challenge inoculum and the denominator indicates the number of mice in the group.

sented in detail in order to indicate the type of mortality distribution usually found in mice which possess different degrees of resistance to infection with scrub typhus. Elsewhere in this report tabular data are given in a more condensed form and include only values for the 50 per cent lethal titers in normal and vaccinated mice and the immunity index; however, each index is based on titrations similar to those illustrated in Table II.

A summary of experiments done with Imphal vaccines is found in Table III. It is evident that the present Imphal vaccines were comparable in potency to those prepared previously (16); they protected mice against 1,000 LD of the homologous strain. Such vaccinated mice displayed a variable degree of resistance when challenged with heterologous strains of *R. tsutsugamushi;* how-

ever, in no instance was the immunity index as great as when the Imphal strain was used. It does not appear feasible with the data available to attempt to arrange sharp levels of immunity when considering resistance to heterologous strains. Instead it seems preferable to consider the results as indicating a broad spectrum with various levels of cross-protection, each of which shades into the other. Thus, Imphal vaccine induced appreciable resistance to the Karp and Kostival strains, slight to wild rat 235, but none to Seerangayee, Pescadores, or mite 21. While the results in general were consistent, divergent data were obtained in the two experiments in which the Volner strain was used

TABLE III

Resistance of Mice Immunized with Imphal Vaccines

Challenge rickettsial strain	Mice injected with								
	Vacc. 1–2			Vacc. 3–4			Vacc.1 –2–5		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.	
Imphal 8	6.1‡	2.5	3.6	6.9	3.4	3.5	6.8	3.7	3.1
Imphal 8				5.8	2.7	3.1	7.0	3.8	3.2
Karp				8.2	6.0	2.2	8.1	6.9	1.2
Kostival	6.7	4.4	2.3	7.1	5.4	1.7			
Wild rat 235							4.3	3.2	1.1
Volner				6.0	3.9	2.1	5.9	5.4	0.5
Seerangayee				6.8	6.5	0.3	6.8	6.6	0.2
Pescadores							7.2	7.2	0.0
Mite 21				6.6	6.7	−0.1			

* See footnote to Table II.

‡ The exponential values of the 50 per cent lethal end point titers are expressed as positive numbers.

for challenge (Table III). It is not unlikely that in the zone of partial immunity secondary factors such as the general health of the test mice prior to challenge have considerable effect on the final result and may account for such discrepancies.[2]

Mice immunized with vaccines prepared against the Karp strain were more resistant to infection with the homologous organism than to infection with the heterologous strains (Table IV). It is evident from the tabular data that the

[2] Vaccinated animals are not solidly immune to challenge even with the homologous agent. They frequently show signs of active infection which persist for a number of days, and during periods of illness the margin of vitality between death and recovery is narrow. The use of occasional groups of mice with inapparent enzootic infections in certain of our experiments may have weighted the balance toward death of the mice and this resulted in lower indices for the test assay.

two vaccines which had indices above 3.0 with the homologous strain afforded some protection against infection with Volner, Kostival, and Imphal strains, little if any against the Seerangayee, and none against mite 21 strain. It is of interest to compare these results with those obtained with vaccine lot 8-9-10-11, which had indices of 2.0 and 0.9 in duplicate tests with the homologous strain (Table IV). This relatively poor vaccine[3] induced no resistance to any of the heterologous strains.

The results of experiments with Kostival vaccines are summarized in Table V. It is evident that the vaccine protected as well against wild rat 235 as against the homologous strain. Furthermore, it induced considerable resist-

TABLE IV

Resistance of Mice Immunized with Karp Vaccines

Challenge rickettsial strain	Mice injected with								
	Vacc. 1			Vacc. 6-7			Vacc. 8-9-10-11		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.	
Karp	8.6*	4.6	4.0	7.9	4.7	3.2	7.9	5.9	2.0
Karp				8.0	4.6	3.4	7.9	7.0	0.9
Volner				6.6	4.2	2.4	6.7	6.6	0.1
Kostival	7.9	6.2	1.7	7.2	5.2	2.0	7.3	7.1	0.2
Imphal 8				5.8	4.1	1.7			
Seerangayee				7.4	6.4	1.0	6.9	6.6	0.3
Mite 21				6.7	6.5	0.2			
Pescadores							7.0	7.1	−0.1
Wild rat 235							3.2	3.7	−0.5

* See footnotes to Tables II and III.

ance to infection with Imphal, Karp, and Volner organisms but none against Seerangayee, mite 21, or Pescadores. Here again some variation occurred in the indices obtained with given heterologous strains in experiments with different batches of vaccine, nevertheless, the trends of the results were consistent.

Pertinent data on the experiments in which mite 21 vaccines were employed are summarized in Table VI. It is immediately obvious that none of the mite 21 preparations was able to induce appreciable resistance to infection with

[3] This pooled material had been stored in the fluid state at 5°C. for about 6 months when the assay was begun; each of its constituent vaccines had been prepared from tissues having a sufficiently high infective titer to suggest that the immunogenic potency would be satisfactory (16). Because of the low potency of the pool we assumed that deterioration had occurred during storage. The results of controlled tests (18) indicated the validity of this assumption and showed that stability on storage could be attained with greater regularity when scrub typhus vaccines were lyophilized.

the mite 21 strain. Nevertheless, these materials were tested with heterologous strains for two reasons: (1) each lot had been prepared from highly infectious material, titer $10^{-7.5}$ to $10^{-8.2}$, and hence contained amounts of rickettsial substances which we thought should have been sufficient to immunize, and, (2) none of the vaccines prepared from Imphal-, Kostival-, or Karp-infected tissues induced even slight resistance to infection with the mite 21 strain. The results were nonetheless unexpected in that the mite 21 vaccines induced as great or greater resistance to infection with wild rat 235, Kostival, Imphal, and Karp strains than to the homologous organism. Indeed, respective indices of 3.5

TABLE V

Resistance of Mice Immunized with Kostival Vaccines

Challenge rickettsial strain	Mice injected with											
	Vacc. 2			Vacc. 5–6			Vacc. 7–8			Vacc. 9–11		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.		Control	Vacc.	
Kostival.....	7.2*	3.9	3.3	6.7	3.0	3.7	7.2	3.4	3.8	7.7	5.6	2.1
Kostival.....				6.5	4.0	2.5						
Wild rat 235...							5.2	1.6	3.6			
Imphal.....				6.1	3.3	2.8				6.9	5.2	1.7
Karp.....	8.4	5.8	2.6	7.7	6.7	1.0						
Volner.....				6.0	4.1	1.9	6.4	5.7	0.7			
Seerangayee...				6.7	6.3	0.4						
Mite 21.....				6.7	7.0	−0.3	6.9	6.9	0.0			
Pescadores....							7.5	7.7	−0.2			

* See footnotes to Tables II and III.

and 3.2 were obtained with the wild rat 235 and Kostival strains in mice immunized with vaccine 4-5 while values of 0.9 and 1.1 resulted in the duplicate test in which the mite 21 strain was used for challenge. In the single experiments in which the Pescadores, Volner, and Seerangayee strains were used for challenge no resistance was noted.

Summary of Results of Cross-Vaccination Experiments.—In order to facilitate a comparison of the antigenic relationships established by the present cross-vaccination experiments, an interpretative summary is given in Table VII. Only those Imphal, Karp, and Kostival vaccines mentioned in Tables III to V which had immunity indices of 3.0 or greater with the homologous organism were considered in compiling the summary. Since none of the mite 21 vaccines gave a high index value with the homologous strain, no selection was applied to the data in Table VI. Indices obtained in several different tests with comparable materials were averaged and when such values were 2.5 or greater the

degree of cross-reaction was arbitrarily designated as ++, similarly, average values from 1.5 to 2.4 were designated as +, values of 0.5 to 1.4 as + −, and those of less than 0.5 as zero for Table VII.

TABLE VI

Resistance of Mice Immunized with Mite 21 Vaccines

Challenge rickettsial strain	Mice injected with								
	Vacc. 1			Vacc. 2-3			Vacc. 4-5		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.	
Mite 21	7.0*	6.3	0.7	6.7	6.2	0.5	7.2	6.3	0.9
Mite 21				7.8	6.4	1.4	6.8	5.7	1.1
Wild rat 235							5.0	1.5	3.5
Imphal							6.5	3.7	2.8
Kostival				7.0	5.5	1.5	7.9	4.7	3.2
Karp	8.2	7.3	0.9	8.2	6.5	1.7	8.1	6.4	1.7
Pescadores							7.1	6.5	0.6
Volner				6.4	6.3	0.1			
Seerangayee				6.8	6.9	−0.1			

* See footnotes to Tables II and III.

TABLE VII

Interpretative Summary of Results of Cross-Vaccination Tests with Strains of R. Tsutsugamushi

Challenge rickettsial strain	Vaccine			
	Imphal	Karp	Kostival	Mite 21
Imphal	++	+	++	++
Karp	+	++	+	+−
Kostival	+	+	++	+
Wild rat 235	+−		++	++
Volner	+−	+	+−	0
Mite 21	0	0	0	+−
Pescadores	0		0	+−
Seerangayee	0	0	0	0

See text for explanation of table.

The data indicate that the Imphal, Kostival, and Karp strains are rather closely related to one another. Furthermore, each of these has one or more immunogenic factors in common with the Volner strain and the first two, at least, also possess components similar to those occurring in wild rat 235. The mite 21 strain contains antigenic structures found in the Imphal, Kostival, Karp, and wild rat 235 organisms, and, in addition, a factor common to itself

and the Pescadores agent. However, the mite 21 organism is peculiar in that vaccines prepared from it appear deficient in substances necessary for inducing good protection against itself. It is particularly striking that vaccines against none of the four agents induced resistance to the Seerangayee organism, hence on the basis of this method of testing, these strains appear entirely unrelated. Furthermore, it is apparent that there is no correlation between the geographical origins of the agents employed (see Materials and methods) and the relationships established by these studies.

Efficacy of a Bivalent Vaccine.—Since the monovalent vaccines prepared from four strains of *R. tsutsugamushi* each failed to induce protection against certain strains of this agent, it appeared desirable to test the immunizing capacity of a

TABLE VIII

Resistance of Mice Immunized with a Bivalent (Karp-Mite 21) Vaccine

Challenge rickettsial strain	Mice injected with								
	Monovalent Karp (diluted 1:2)			Monovalent mite 21 (diluted 1:2)			Bivalent Karp and mite 21		
	Titer in		Immunity index*	Titer in		Immunity index	Titer in		Immunity index
	Control	Vacc.		Control	Vacc.		Control	Vacc.	
Karp	8.2*	5.5	2.7				8.2	5.7	2.5
Mite 21				7.4	6.6	0.8	7.4	6.5	0.9
Seerangayee							6.5	6.3	0.2

Karp vaccine 12 and mite 21 vaccine pool 4-5 were mixed in equal proportions for the bivalent vaccine. Each monovalent vaccine diluted with an equal volume of saline was used for a comparative test.

* See footnotes to Tables II and III.

bivalent vaccine. Accordingly, a mixture of equal volumes of a mite 21 and a Karp vaccine was used to immunize a group of mice. In addition, other groups of mice received the mite 21 or Karp vaccine diluted with equal volumes of physiological saline solution. Subsequently the animals which had been injected with the diluted monovalent vaccines were challenged with the homologous organism while those immunized with the bivalent vaccine were divided into three groups and infected with either mite 21, Karp, or Seerangayee strains of rickettsiae. The results of this experiment are presented in Table VIII.

It is evident that resistance induced by the bivalent vaccine was essentially the same as that induced individually by each of its component parts. Since neither component alone elicited immunity to the Seerangayee strain, it was to be expected that the mixture would also fail to immunize against this heterologous strain. The same reasoning might explain the lack of augmentation of resistance to the mite 21 agent in the mice receiving the mixture. However, it had been anticipated that the bivalent material would, at least, provide

increased protection against Karp since the results of earlier experiments had indicated that mite 21 vaccine induced some immunity to the Karp strain.

Resistance of Convalescent Mice to Inoculation of Homologous and Heterologous Strains of R. tsutsugamushi.—It has been demonstrated repeatedly that animals infected with one strain of *R. tsutsugamushi* are resistant to infection with heterologous strains of this agent. Each of the strains used in the present work had been previously tested in this respect and found to respond in the usual manner (see Table I, under Materials and methods). Nevertheless, in view of the differences shown to exist among the eight strains by means of the cross-vaccination tests discussed above, it seemed desirable to recheck the identity of the strains at the termination of the present study. Accordingly, mice convalescent from infection induced by the subcutaneous injection of small amounts of Kostival rickettsiae were segregated in groups of five and injected intraperitoneally with 10, 100, or 1,000 minimal lethal doses of organisms of the Kostival, Imphal, Karp, mite 21, Volner, Pescadores, or Seerangayee strains. Convalescent animals survived inoculation with the various challenge materials, hence, all of these strains appeared characteristic of *R. tsutsugamushi* when reexamined in a cross-immunity test.

DISCUSSION

Formalin-inactivated vaccines prepared from tissues of rats infected with the Imphal, Karp, Kostival, or mite 21 strains of *R. tsutsugamushi* induced resistance in mice against infection with the homologous as well as certain heterologous strains of the organism. However, none of the vaccines was capable of eliciting protection against all eight strains of *R. tsutsugamushi* employed in the present work. These results, which add evidence to that already obtained by other methods (11–14), warrant the conclusion that significant differences in antigenic structure exist among the organisms now classified as *R. tsutsugamushi* even though infection with one strain renders animals immune to reinfection with other strains of the agent. Despite these differences, at the present time it does not appear desirable to indulge in taxonomic speculation regarding division of the subgenus *R. tsutsugamushi*. However, it may be noted that while cross-immunity is exhibited by animals which recover from infection with *R. prowazeki* and *R. typhi*, or from infections with *Dermacentroxenus rickettsi* and *D. coroni*, nevertheless, vaccines prepared against one member of the pairs of agents afford comparatively little protection against the other (25–27).

The present observations suggest that certain strains of *R. tsutsugamushi* may be more broadly antigenic than others. If this is true, then a search is warranted for an organism possessing the entire antigenic mosaic of the subgenus since it might yield a vaccine capable of protecting man against naturally occurring infection with scrub typhus. It should be borne in mind, however, that those strains which appear to possess more antigenic constituents than

others may consist actually of a mixture of several strains. Such mixtures might be obtained unwittingly in the laboratory by combining infectious material from several sources. Thus, the Imphal 8 strain was derived from the pooled bloods of five patients and the mite 21 agent from a pool of over 250 mites. Furthermore, the possibility exists that the rickettsiae recovered from a single naturally infected rodent may represent a biological mixture of strains. This idea receives some support from the observations of Audy (28) on one of the mite vectors in the Imphal area; he states, ". . . in many populous colonies of wild rats the turnover of *Trombicula diliensis* may well amount to some 5,000 larvae per rat per annum." The possibility of a dual infection in such an infested rat is not unlikely.

CONCLUSION

Antigenic differences among strains of *R. tsutsugamushi* are sufficiently great that vaccines prepared from certain strains fail to induce resistance in mice to infection with other strains. Although the results of cross-vaccination tests indicate varying degrees of relationship between a number of the strains, there is no correlation between source of the rickettsia and antigenic pattern of the agent.

BIBLIOGRAPHY

1. Nagayo, M., Miyagawa, Y., Mitamura, T., Tamiya, T., Satu, K., Hazato, H., and Imamura, A., *Japan. J. Exp. Med.*, 1931, **9**, 87.
2. Lewthwaite, R., and Savoor, S. R., *Brit. J. Exp. Path.*, 1936, **17**, 448.
3. Kawamura, R., Kasahara, S., Toyama, T., Nisinarita, F., and Tsubaki, S., *Kitasato Arch. Exp. Med.*, 1939, **16**, 93.
4. Lewthwaite, R., and Savoor, S. R., *Brit. J. Exp. Path.*, 1940, **21**, 117.
5. Kouwenaar, W., and Wolff, J. W., *Proc. 6th Cong., Pacific Science Assn.*, 1939, Berkeley, University of California Press, 1942, **5**, 633.
6. Blake, F. G., Maxcy, K. F., Sadusk, J. F., Jr., Kohls, G. M., and Bell, E. J., *Am. J. Hyg.*, 1945, **41**, 243.
7. Kohls, G. M., Armbrust, C. A., Irons, E. N., and Philip, C. B., *Am. J. Hyg.*, 1945, **41**, 374.
8. Topping, N. H., *Pub. Health Rep., U. S. P. H. S.*, 1945, **60**, 945.
9. Zarafonetis, C. J. D., Snyder, J. C., and Murray, E. S., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 240.
10. Smadel, J. E., Rights, F. L., Jackson, E. B., and Bennett, B. L., unpublished data.
11. Bengtson, I. A., *Pub. Health Rep., U. S. P. H. S.*, 1945, **60**, 1483.
12. Bengtson, I. A., *Pub. Health Rep., U. S. P. H. S.*, 1946, **61**, 887.
13. Bell, E. J., Bennett, B. L., and Whitman, L., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 134.
14. Smadel, J. E., Jackson, E. B., Bennett, B. L., and Rights, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 138.
15. Fulton, F., and Joyner, L., *Lancet*, 1945, **2**, 729.

16. Smadel, J. E., Rights, F. L., and Jackson, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61,** 308.
17. Plotz, H., Bennett, B. L., and Reagan, R. L., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61,** 313.
18. Rights, F. L., and Smadel, J. E., unabridged dissertation, "Studies on scrub typhus (tsutsugamushi disease). III. The heterogenicity of certain strains of *R. orientalis* as determined by cross-vaccination studies." Filed with the Library of the College of Physicians and Surgeons, Columbia University, New York.
19. Smadel, J. E., Rights, F. L., and Jackson, E. B., *J. Exp. Med.*, 1946, **83,** 133.
20. Kawamura, R., and Nakamura, R., *Kitasato Arch. Exp. Med.*, 1937, **14,** 75.
21. Berge, T. O., personal communication.
22. Lewthwaite, R., and Savoor, S. R., *Brit. J. Exp. Path.*, 1936, **17,** 1.
23. Philip, C. B., Woodward, T. E., and Sullivan, R. R., *Am. J. Trop. Med.*, 1946, **26,** 229.
24. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
25. Castaneda, M. R., *Brit. J. Exp. Path.*, 1941, **22,** 167.
26. Topping, N. H., Bengtson, I. A., and Henderson, R. G., *Nat. Inst. Health Bull. No. 183*, Washington, D. C., United States Government Printing Office, 1945, 57–64.
27. Davis, G. E., and Parker, R. R., *Pub. Health Rep., U. S. P. H. S.*, 1934, **49,** 423.
28. Audy, J. R., A review of investigations on the epidemiology of scrub typhus 1945–46, in Scrub typhus investigations in S. E. Asia, Part 1, General Report, Army Medical Directorate, The War Office, London, 1947, 28–54.