STUDIES ON SCRUB TYPHUS

I. Soluble Antigen in Tissues and Body Fluids of Infected Mice and Rats*

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(Received for publication, November 13, 1945)

Serologically active soluble substances have been demonstrated in tissues infected with a number of viral and rickettsial agents. While viruses have been studied more exhaustively than rickettsiae as regards the occurrence of such antigens, nevertheless soluble substances have been found associated with four members of the latter groups of agents, namely, the organisms of epidemic and murine typhus (1–3), of Rocky Mountain spotted fever (3), and of fièvre boutonneuse (3).

The present paper describes experimental results which indicate that the tissues and body fluids of certain rodents infected with scrub typhus contain a specific serological substance that is considerably smaller than the rickettsial organism and which may be designated as a soluble antigen. The excellent and timely articles of Blake, Maxcy, Sadusk, Kohls, and Bell (4) and of Kohls, Armbrust, Irons, and Philip (5) should be consulted by those interested in various aspects of the subject of scrub typhus.

Materials and Methods

Strains of R. orientalis.—The Imphal No. 8 and Calcutta strains (6) of *R. orientalis* were employed in the present studies. These organisms which were originally isolated late in 1943 by Major M. T. Parker, R.A.M.C., and Dr. S. R. Savoor, were obtained early in 1944 from Major C. J. D. Zarafonetis, M.C., in the 6th and 7th passages in rabbits. The agents were

* Experimental work discussed here was done in the Virus Division, First Medical General Laboratory, U. S. Army. The results were summarized in reports to the Director of the United States of America Typhus Commission which were submitted on August 19, 1944, and March 31, 1945. These were entitled "Report of laboratory studies on two Indian strains of scrub typhus" and "A specific soluble antigen in blood of animals experimentally infected with scrub typhus."

subsequently maintained by serial passage in the yolk sacs of embryonated eggs which had been incubated at 37°C. for 6 days prior to infection. Seed material for inoculation of animals consisted of lightly centrifuged 10 per cent suspensions of fresh yolk sacs which were harvested on the 8th to 10th days after infection with the 12th to the 43rd serial passages of the strains. Inoculated eggs were incubated at 35°C. The infective titers of such seed suspensions generally ranged between $10^{-8.0}$ and $10^{-8.5}$. In the present work, seed inoculum was considered unsatisfactory for intravenous injection of mice and rats when its infective titer was less than $10^{-8.0}$. Experiments in which such seed was used have been omitted from consideration in this report.

Materials from Mice, White Rats, and Cotton Rats.—Tissues and body fluids used in the present serological studies were generally obtained from mice and rats which were injected by the intravenous route with highly infectious suspensions of yolk sac. Mice received 0.5 cc. amounts of inoculum while young adult white rats were given 1.0 or 2.0 cc. quantities intravenously. It was difficult to inject material into the tail veins of young adult cotton rats, consequently 1.0 cc. volumes of the infectious suspensions were injected intracardially into some of these animals. All three species of rodents died or were moribund on the 4th to 6th days following the intravenous administration of 10 per cent suspensions of yolk sac that had infectious titers of $10^{-8.0}$ or greater. Macroscopic changes in such animals were limited essentially to patchy hemorrhagic pneumonia, extensive fibrinoserous pleural effusion, and splenic enlargement. Enlargement of the spleen was more prominent in white mice and rats than in cotton rats. Cardiac blood and pleural fluid were collected by aspiration with a syringe and needle from groups of moribund animals; care was taken to avoid contaminating one type of specimen with the other. 0.5 to 1.5 cc. of pleural fluid were generally obtained from mice, and 2.0 to 6.0 cc. from rats. Lungs and spleens were removed from mice and rats when the animals were moribund or within 1 to 2 hours after death.

After these tissues were ground in a mortar in the presence of small amounts of alundum, a 10 per cent suspension in physiological saline solution buffered at pH 7.6 was prepared. This was centrifuged horizontally at 2,000 R.P.M. and the resultant sediment discarded. Portions of these tissue suspensions were taken immediately for determination of their infectivity.

In certain instances body fluids and tissues were collected from mice that sickened and died 1 to 3 weeks after infection. The prolonged incubation period and protracted course of the disease were induced by injecting mice intraperitoneally with varying concentrations of *R. orientalis*.

Pooled serum from hyperimmune mice was employed in all complement fixation tests for titration of antigen in the materials under investigation. Such serum was obtained from mice which were infected originally by the subcutaneous route with a suspension of mouse spleen or lung tissue which contained 10^3 or 10^4 M.L.D. of *R. orientalis* (determined by intraperitoneal titration in mice); 4 weeks later the mice were injected intraperitoneally with suspensions of fresh mouse tissue containing 10^4 to 10^6 M.L.D. of the agent. 10 days following the second injection, hyperimmune serum was collected and stored in quantities of 1 to 2 cc. at −20°C.; the serum was inactivated at 56°C. immediately prior to use. Groups of mice which provided the immune serum were infected and reinoculated with either the Imphal or Calcutta strains; in some instances mice were infected with one and reinoculated with the other strain.

Preparation of Antigens.—Fresh suspensions of infected rodent tissues, prepared in the manner described above, were treated promptly with sufficient quantities of U.S.P. formaldehyde solution and of merthiolate to bring the final concentrations to 0.1 per cent and 0.01 per cent, respectively. They were then stored at +5°C. for 5 to 7 days in order to permit inactivation of the rickettsiae to take place. Certain of the earlier preparations were tested for complement-fixing activity without further treatment other than removal of coarse sediment which appeared during storage. These materials were designated as "crude antigens."

Later suspensions were extracted, prior to serological testing, in the following manner with ethyl ether. Equal volumes of tissue suspension and ethyl ether were mixed in a separatory funnel and left at room temperature for 4 to 6 hours. During this period of time the mixture was shaken vigorously on several occasions. The aqueous phase was separated from the ether and emulsion layers and centrifuged at 2,000 R.P.M. for 5 minutes. The supernatant fluid was freed of ether by storage overnight at 5°C. in a cotton-stoppered flask; to promote evaporation just sufficient fluid was employed to form a shallow layer on the bottom of the container. Such formalinized ether-extracted crude antigens were designated as "stock antigens."

Pooled sera and pleural fluids from groups of infected animals were tested for serological activity within a few hours after they were obtained; it was important to store the latter substances for a short time in order to permit the fibrin to clot, after which it could be removed. In order to destroy the infectivity of both types of fluid they were inactivated at 56°C. for ½ hour immediately before testing.

Intact rickettsiae were removed from certain of the formalinized tissue suspensions and from fresh body fluids by centrifugation in lusteroid tubes with a diameter of 1 cm. at 18,400 R.P.M. for 1½ hours in a specially adapted multispeed angle head of a refrigerated International centrifuge (7).

Complement Fixation Technique.—The technique employed in the present work was essentially identical with that used at the Army Medical School for studies on other rickettsial diseases (8). Two full units of complement and two units of antibody (usually a 1/64 dilution of hyperimmune mouse serum contained the requisite amount of antibody) were added to serial dilutions of the fluid to be tested for antigen. Tubes containing the mixtures were stored overnight at 5°C. and the hemolytic system was added the next morning. The tests were read in the usual manner after incubation at 37°C. for 30 minutes. In other instances in which titrations of complement-fixing antibody were performed two units of scrub typhus antigen were used in the test.

Determination of Infective Titer.—The infectivity of various materials was determined in the following manner. The original 10 per cent suspension of yolk sac or of rodent tissue, prepared in buffered physiological saline solution, was lightly centrifuged, after which serial tenfold dilutions were made in a mixture containing one part normal horse serum and nine parts physiological saline solution. Groups of 3 to 6 mice were injected intraperitoneally with 0.2 cc. amounts of each dilution of the material to be tested. In some instances clotted blood of mice was ground in a mortar and lightly centrifuged after which the supernatant fluid was titered for infectivity; in others heparinized plasma was titered. Infected animals were observed for a period of 21 days and deaths were recorded. The infective titers were estimated by applying the 50 per cent endpoint method to the resultant data; only animals dying between the 6th and 21st days were placed in the positive group.

The Swiss strain of mice from the British Agricultural Research Station at Compton, England, was used exclusively in titration experiments until the terminal portion of the work. Then the Farm strain was employed in a few instances; this was an inbred line of mice of varying colors, predominantly brown, which was maintained at Compton. Swiss mice were somewhat more susceptible than Farm mice to *R. orientalis* administered by the intraperitoneal route. For example, in several comparative experiments the 50 per cent endpoint lethal titer of a given material was approximately 1.0 log dilution greater when determined in Swiss mice. Moreover, the number of survivors among groups of mice receiving multiple M.L.D. of rickettsiae was always greater when the Farm strain was employed. Such surviving Farm mice regularly showed characteristic macroscopic evidence of infection when sacrificed on the 21st day, and usually rickettsiae could be identified microscopically in stained impression smears of their spleens. In those experiments in which Farm mice were used, the infective titer was based on a positive group that included not only the mice which succumbed but also

those which survived 21 days and showed rickettsiae on microscopic examination. This method of estimating the endpoint raised the titer about 1 log above that which was obtained when only dead Farm mice were included in the positive group.

EXPERIMENTAL

Bengtson (9) reported the presence of a specific complement-fixing antigen of scrub typhus in preparations of infected yolk sac. Eventually we also were able to demonstrate such an antigen in egg materials but during the first part of our work with *R. orientalis* we were unsuccessful. These early failures we ascribed to the relative paucity of rickettsiae in the yolk sacs infected with the passages of our strains then available. Search for other sources of scrub typhus antigen led to the experiments discussed in this paper.

TABLE I

Effect of Ether Extraction on Complement-Fixing Antigen Prepared from Infected Mouse Spleen

Antigen		Serum	Dilution of serum					A.C.* serum 1/16	A.C.* antigen	
No.	Treatment		1/16	1/32	1/64	1/128	1/256		1/1.5	1/3.0
Imphal 2 (used at 1/3 dilution)	Crude	Immune mouse pool No. 900	4	4	4	4		0	±	0
		Normal mouse pool	4	4	2	1		0		
Imphal 2 (used at 1/1 dilution)	Ether-extracted	Immune mouse pool No. 900	4	4	4	4	0		0.5 cc. 1/1	0.25 cc. 1/1
		Normal mouse pool	0	0	0	0	0		0	0

* A.C. indicates anticomplementary control.

Demonstration of Complement-Fixing Antigen in Tissues of Infected Mice

Crude suspensions of lung and spleen tissue from moribund mice, prepared in the manner described above, were capable of fixing complement both in the presence of hyperimmune mouse serum and of normal mouse serum. Thus, the demonstration of any specific scrub typhus reaction was obscured by the occurrence in mouse serum of a material similar to the so called natural antibody which Kidd and Friedewald (10) described in rabbit serum. The substance in suspensions of mouse tissue which reacted with normal serum was readily removed by extracting the suspensions with ethyl ether in the manner mentioned in the section on Materials and methods. Treated suspensions fixed complement with immune but not with normal mouse serum (Table I). After the value of extraction of tissue suspensions was established, all crude tissue antigens were invariably treated with ether before being tested.

It was promptly demonstrated that antigens prepared from mice infected with the Imphal or Calcutta strain fixed complement equally well with pooled sera from mice immunized with either one of the strains, or with both (Table II). Thus, the results indicated that the two strains with which we worked were antigenically similar, indeed, on the basis of these data alone they were indistinguishable. Further evidence for the close relationship of the strains was forthcoming from cross-immunity tests which were performed in our laboratory at about the same time. The results of these tests showed that mice which recovered from an inapparent infection induced by subcutaneous injection of

TABLE II

Demonstration of Common Antigen in Tissues Infected with Imphal and Calcutta Strains

Antigen*		Mouse Serum		Dilution of serum				
Strain	No.	Strain	No.	1/16	1/32	1/64	1/128	1/256
Imphal	9	Imphal	652	4	4	4	4	±
		Calcutta	899	4	4	4	4	2
		Imphal-Calcutta	900	4	4	4	4	2
		Normal	660	0	0	0	0	0
Calcutta	1	Imphal	652	4	4	4	4	3
		Calcutta	899	4	4	4	4	3
		Imphal-Calcutta	900	4	4	4	4	1
		Normal	600	0	0	0		
Normal mouse		Imphal	652	0	0	0	0	0
		Calcutta	899	0	0	0	0	0
		Imphal-Calcutta	900	0	0	0	0	
		Normal	901	0	0	0	0	

* Each antigen was an ether-extracted preparation of a formalinized 10 per cent suspension of mouse lung and was used at a 1/1 dilution.

one of the strains were immune to subsequent intraperitioneal inoculation of lethal amounts of *R. orientalis* of the homologous or heterologous strain (reference cited in (4)).

Specificity of the Complement Fixation Reaction in Scrub Typhus

The specificity of the serologically active substance present in ether-extracted suspensions of tissue from mice infected with *R. orientalis* was next investigated.

Mouse lung antigens were tested with serial dilutions of sera of a number of different types. These included: (1) hyperimmune sera from mice prepared in the manner described above,

TABLE III

Specificity of the Complement Fixation Reaction in Scrub Typhus

Typhus group	Serum		Complement fixation titers of serum		
			Epidemic	Murine	Scrub
Scrub	*Mouse*				
	Imphal	Pool 652	0	0	1/256
	Imphal	Pool 1054	0	0	1/128
	Calcutta	Pool 899	0	0	1/256
	Calcutta	Pool 653	0	0	1/64
	Calcutta-Imphal	Pool 900	0	0	1/256
	Guinea pig				
	Imphal	Pool 131	0	0	1/32
	Imphal	Pool 171	N.D.*	N.D.	1/4
	Imphal	Pool 172	N.D.	N.D.	1/8
	Rabbit				
	Imphal	Pool 19	N.D.	N.D.	1/32
	Imphal	Pool 20	N.D.	N.D.	1/8
	Calcutta	Pool 12	N.D.	N.D.	1/16
Epidemic	Spooner		1/256	1/64	0
	Orsini		1/80	N.D.	0
	Human pool, Naples		1/160	0	0
Murine	Guinea pig (Wilmington)		0	1/128	0
	Human 3		1/128	1/256	0
	Human 19/5		0	1/256	0
"Intermediate strains"	Mysore G.P. 44		1/8	1/128	0
	Haffkine G.P. 71		0	1/32	0
	Haffkine G.P. 73		1/32	1/128	0
Vaccinated (epidemic and murine)	J. S.		1/8	1/4	0
	A. L.		1/16	1/8	0
	R. M.		1/16	1/8	0
	J. H.		1/32	1/16	0
	W. W.		1/32	1/8	0
	E. J.		1/16	1/8	0

* N.D. indicates not done.

Ether-extracted suspensions of lung tissue of mice infected with scrub typhus and purified suspensions of epidemic and murine rickettsiae were used as antigens in the tests. See text for other details.

(2) sera from guinea pigs and rabbits which had received several intraperitoneal injections of yolk sac suspension rich in organisms of the Imphal No. 8 or Calcutta strains, (3) sera[1] from guinea pigs convalescent from infection with the Wilmington strain of murine typhus or with

[1] These sera were supplied by Major Janet Niven, R.A.M.C., of the Emergency Vaccine Laboratory, Everleigh, Wiltshire, England.

the Haffkine or Mysore agents which have been considered as intermediate strains of typhus, (4) sera from patients recovered from epidemic and murine[1] typhus, and (5) sera from Army personnel who had been injected repeatedly with epidemic and murine typhus vaccines. The majority of the scrub typhus sera were also tested for antibodies against epidemic and murine typhus. These tests were performed by the technique (8) employed at the Army Medical School with washed rickettsial antigens of epidemic and murine typhus supplied by that institution. No evidence was forthcoming of cross-reactions between scrub typhus antigens and antisera against the other rickettsial diseases; furthermore, scrub typhus antisera failed to fix complement with washed suspensions of epidemic and murine typhus organisms. The results of the tests with materials from scrub, epidemic, and murine typhus and from the so called "intermediate strains" are summarized in Table III.

It can be concluded from the results presented in Table III that the complement-fixing antigen present in preparations of lung tissue of mice infected with scrub typhus is specific for the disease. Similar observations, previously reported by Bengtson (9), likewise led her to conclude that a specific antigen of scrub typhus occurs in yolk sacs infected with the Karp strain of *R. orientalis*.

Scrub Typhus Antigen in Blood and Pleural Fluid of Infected Mice

Smorodintzeff and his coworkers (11) found a specific serological substance in the blood of patients in the pre-eruptive stage of epidemic typhus fever, and devised methods for the early diagnosis of this disease based on the demonstration of the circulating antigen. Experiments were designed to determine whether the specific complement-fixing antigen of scrub typhus occurred in the body fluids of mice infected with *R. orientalis*. The results of a number of these experiments are summarized in Table IV.

It is apparent from the data presented in Table IV that serum and pleural fluid collected from mice which died 4 to 6 days after intravenous injection of suspensions of yolk sac infected with the Imphal No. 8 or Calcutta strains contained appreciable amounts of antigen that fixes complement with scrub typhus antibody. However, the conditions under which this circulating antigen was demonstrable were sharply demarcated. Thus, the route of inoculation of highly potent suspensions of rickettsiae and the duration of the disease were of utmost importance. The protracted illness that resulted when mice were injected intraperitoneally with small amounts of *R. orientalis* was not accompanied by the presence of demonstrable antigen in the body fluids. Even the most rapidly fatal course that followed intraperitoneal infection with our strains, namely 7 days, was associated with the occurrence of only questionable amounts of antigen in the blood. Early experience had revealed that tissues from mice which succumbed between the 12th and 21st days contained no detectable scrub typhus antigen. Similarly, it was now shown that body fluids of mice that die between the 13th and 19th days gave negative results in tests for antigen (Table IV). It will be noted that scrub typhus antibody was

present in small amounts, titers ½ and ¼, in serum of mice moribund on the 15th and 19th days after inoculation (Table IV).

TABLE IV

Scrub Typhus Antigen in Serum and Pleural Fluid of Infected Mice

Antigen		Obtained from mice		Complement fixation tests							
No.	Type	Infected	Died	Dilution of antigen						A.C. control	
				1/2	1/3	1/4	1/6	1/8	1/12	1/2	1/3
		Strain Source Route	*Day*								
170	Pleural fluid	Cal. Y.S. 10^{-1} I.V.	4	4	4	4	4	2	2	0	0
93	Pleural fluid	Imph. Y.S. 10^{-1} I.V.	5	4	4	4	4	4	4	0	0
94	Serum	Same mice as 93	5	4	4	4	4	4	3	0	0
37	Serum	Imph. Y.S. 10^{-1} I.V.	6	4	4	4	4	4	3	4	±
69	Serum	Imph. Y.S. 10^{-1} I.V.	6		4	4	4	4	3		0
73	Serum	Imph. Y.S. 10^{-1} I.P.	7	4	0	0	0	0	0	±	0
82	Serum	Imph. Y.S. 10^{-4} I.P.	13	0	0	0	0	0		0	0
83	Pleural fluid	Same mice as 82	13	0	0	0	0	0		0	0
54	Serum	Imph. Y.S. 10^{-7} I.P.	15	0	0	0	0	0	*	0	0
56	Pleural fluid	Same mice as 54	15	0	0	0	0	0		0	0
70	Pleural fluid	Imph. Y.S. 10^{-8} I.P.	19	±	0	0	0	0		±	0
71	Serum	Same mice as 70	19	0	0	0	0	0	*	0	0

* This serum contained small amounts of antibody which reacted with scrub typhus antigen.

Sera and pleural fluids were inactivated at 56°C. for ½ hour prior to testing for presence of antigens. Scrub typhus immune mouse serum, titer 1/128, was used at 1/64 dilution in the tests. Anticomplementary control tests on each antigen were run in the presence of 1/64 dilution of normal mouse serum.

Scrub Typhus Antigen in Tissues and Body Fluids of Infected White Rats and Cotton Rats

Rats of several species are known to develop low grade or inapparent infections with *R. orientalis*. These include naturally infected wild rats trapped in Malaya (12) and New Guinea (5), and white rats infected in the laboratory (13). Recently, Fulton (14) has shown that cotton rats develop a rapidly fatal disease when infected by the intranasal route, and we have found that white rats respond similarly to intravenous injection of the agent. During the course of other studies information was accumulated concerning the occurrence of scrub typhus antigen in the tissues and body fluids of white and cotton rats. These data, together with certain results obtained in comparable experiments in mice, are summarized in Table V.

Scrub typhus antigen was demonstrated in the serum, pleural fluid, and tissues of white rats and cotton rats injected by the intravenous route with

highly infectious suspensions of yolk sac (Table V). In general, observations made on the two species of rats were similar in all essential respects to those made on mice. The titers of antigen in serum and pleural fluid, and in 10 per cent suspensions of infected organs from the same batches of animals were of the same order of magnitude. However, the actual amount of antigen was always greater in the tissues than in the body fluids since a positive reaction with a $\frac{1}{8}$ dilution of lung suspension, for example, really represented a $\frac{1}{80}$ dilution of tissue.

TABLE V

Comparison of Amounts of Antigen in Serum, Pleural Fluid, Lung, and Spleen of Infected Mice and Rats

Experiment	Serum Complement fixation titer	Pleural fluid Complement fixation titer	Tissue suspensions			
			Lung		Spleen	
			Complement fixation titer	Infection titer	Complement fixation titer	Infection titer
Mouse 26	1/12	N.D.	1/3	$10^{-8.5}$	1/1	$10^{-7.5}$
Mouse 27	1/12	N.D.	1/4	$10^{-7.5}$	1/1	N.D.
Mouse 28	1/12	1/12	1/8	$10^{-8.5}$	1/3	$10^{-8.5}$
White rat 29	1/2	1/3	1/3	$10^{-7.5}$	N.D.	N.D.
White rat 30	1/2	1/3	1/3	$10^{-8.8}$	N.D.	N.D.
Cotton rat 32	N.D.	1/3	1/4	$10^{-7.8}$	1/1	$10^{-7.3}$
White rat 33	1/3	1/2	1/2	$10^{-8.8}$	1/1	$10^{-8.0}$
Cotton rat 34	1/3	1/3	1/6	$10^{-8.0}$	1/1	$10^{-7.8}$
White rat 35	1/8	1/3	1/4	$10^{-9.0}$	1/4	$10^{-8.3}$
White rat 37	1/12	1/4	1/8	$10^{-8.3}$	1/6	$10^{-7.3}$

Sera and pleural fluid to be tested for antigen were inactivated at 56°C. for ½ hour. The formalinized 10 per cent tissue suspensions were extracted with ether prior to testing. The 29th to 39th passages in yolk sac of the Imphal No. 8 strain were used to infect the animals.

Data on the infective titers of the various organs that were used to prepare complement-fixing antigens are included in Table V. Within the relatively narrow ranges covered by the results it was not possible to correlate the infective and serological titers sufficiently closely so that one could be used as a basis for estimating the other. The results of earlier experiments with mice had indicated that complement-fixing antigen was not demonstrable[2] in tissues with an infective titer of less than 10^{-7}.

[2] In two instances suspensions of mouse lung had complement-fixing titers of ¼ and ⅛ while their infective titers were only $10^{-6.0}$ and $10^{-6.5}$, respectively. In both cases the tissues had been emulsified by prolonged shaking in a bottle containing considerable quantities of glass beads and alundum, mesh No. 60. It was subsequently demonstrated that the infective titer obtained when tissues were ground by this method was several log dilutions below that of portions of the same organs which were triturated in a mortar.

Demonstration of Specific Soluble Substance of Scrub Typhus

Practically all of the specific complement-fixing material present in our ether-extracted preparations of infected mouse tissue occurred in the form of a "soluble antigen." The results obtained in two illustrative experiments are summarized in Table VI. These indicate that the serologically active substance was not appreciably sedimented by high speed centrifugation; *i.e.*, spinning in the cold in lusteroid tubes with a diameter of 1 cm. in an angle head at 18,400 R.P.M. for 1½ hours. Centrifugation under these conditions was more than adequate for the sedimentation of bacillary forms of rickettsiae or even of elementary bodies of such viruses as vaccinia or psittacosis (7). It may be pointed out that microscopically recognizable forms of *R. orientalis* were not observed in the formalinized ether-extracted preparations of infected mouse tissue or in the ultrasediments obtained from them. Nevertheless they had been seen in smears of the original tissues and in the freshly prepared suspensions of the organs. The failure to detect complement-fixing antigen in the ultrasediment was not unexpected in view of the dearth of intact rickettsiae. The observations just reported offered no direct evidence for the natural occurrence in the living animal of the specific soluble substance of scrub typhus. It is obvious that manipulations employed during preparation of antigen from tissue may have resulted in the release of complement-fixing substance from damaged rickettsiae.

Information on the natural occurrence of soluble antigen was derived from studies of blood of infected animals. It was found in experiments undertaken for another purpose that the infective titer of blood of mice which were moribund 4 to 6 days after inoculation was relatively low when compared with that of tissues from such animals. Thus, in seven instances the titers of mouse blood ranged between $10^{-3.3}$ and $10^{-5.6}$, with the majority of the endpoints falling between 10^{-4} and 10^{-5}. Despite these findings, serum of such animals contained appreciable amounts of specific complement-fixing antigen (Tables IV, V, and VII). The sharp difference between blood and tissues as regards the relationship of infective and complement-fixing titers led us to suspect that the serologically active substance in blood existed for the most part in the form of a soluble antigen. The results of ultracentrifugal experiments designed to test this hypothesis are summarized in Table VII. The serologically specific substance in blood of mice and white rats was not sedimented by spinning at 18,400 R.P.M. for 1½ hours. The preparation of serum specimens required little or no manipulation that might be expected to disrupt rickettsiae even if the organisms had been present in sufficient quantities to provide such amounts of complement-fixing antigen. Therefore, these results indicate that the specific soluble antigen of scrub typhus does occur in the infected host during life. No attempts were made to determine how much of the serologically

TABLE VI

Demonstration of Specific Soluble Substance in Scrub Typhus Antigens

Typhus strain	Type of antigen	Complement fixation tests								
		Dilution of antigen						Antigen A.C.		
		1/1	1/2	1/3	1/4	1/6	1/8	1/1	1/2	1/3
Calcutta (mouse lung)	Stock			4	4	3	1			0
	18,400 supernatant	4	4	4	4	2	0	0	0	
	18,400 sediment	0	0	0	0	0	0	0	0	
Imphal (mouse spleen)	Stock	4	4	4	3	0	0	0	0	
	18,400 supernatant	4	4	4	3	0	0	0	0	
	18,400 sediment	0	0	0	0	0	0	0	0	

Imphal-Calcutta mouse serum pool 900 was used at 1/32 dilution in the above tests.

TABLE VII

Specific Soluble Antigen of Scrub Typhus in Blood of Infected Mice and Rats

Antigen	Serum			Complement fixation test Dilution of antigen				
	Disease	Source	Dilution	1/3	1/4	1/6	1/8	1/12
Mouse serum, pool 43	Scrub	Mouse	1/64	4	4	4	4	0
	Epidemic	G.P.	1/40	0	0	0	0	0
	Murine	G.P.	1/40	0	0	0	0	0
	Normal	G.P.	1/40	0	0	0	0	0
	Normal	Mouse	1/64	0	0	0	0	0
Serum 43 after centrifugation at 18,400 R.P.M. for 1½ hours	Scrub	Mouse	1/64	4	4	4	4	0
	Normal	Mouse	1/64	0	0	0	0	0
White rat serum, pool 171	Scrub	Mouse	1/64	4	4	4	4	3
	Normal	Mouse	1/64	2	0	0	0	0
Serum 171 after centrifugation at 18,400 R.P.M. for 1½ hours	Scrub	Mouse	1/64	4	4	4	4	1
	Normal	Mouse	1/64	1	0	0	0	0

Sera employed as antigens were inactivated at 56°C. for ½ hour before testing. Donor mice and white rats were moribund when bled on 5th day after I.V. injection of suspension of Imphal yolk sac.

Antisera had the following titers when tested with homologous antigens; scrub typhus hyperimmune mouse serum, 1/128; epidemic typhus immune guinea pig serum, 1/128; murine typhus immune guinea pig serum, 1/128.

active material present in fresh infectious tissue occurred in the form of a soluble antigen and how much was associated with the intact virulent rickettsial organisms.

Observations on the Stability of Scrub Typhus Antigen

Certain information was collected on the stability of the complement-fixing antigen of scrub typhus. This will be mentioned for, even though incomplete, it bears directly on several practical aspects of the problem of scrub typhus.

The antigen present in serum and pleural fluid of mice and rats was relatively stable. For example, it was demonstrable after heating at 56°C. for ½ hour. This treatment was applied to all such specimens tested, hence no data became available on the amount of antigen, if any, which was lost by heating. Serum and pleural fluid had the same complement-fixing titer when tested immediately after collection, and after storage for several weeks at +5°C. or at −20°C.; the specimens were stored in the undiluted state and were not inactivated. Furthermore, no reduction in titer was noted in samples of each type of fluid which were rehydrated after having been dried from the frozen state.

The complement-fixing titer of ether-extracted suspensions of infected tissue decreased at a variable rate in different preparations during storage at 5°C. Six of the twelve antigens that were tested at intervals showed a moderate decline in titer at the end of 1 month, for example, ⅛ to ¼. The remaining six had lost most of their activity at the end of 2 weeks and were inactive after a month. In certain instances residual ether, left in the suspensions after extraction, had been removed rapidly by warming the material at 37°C. for 1 to 2 hours; antigens prepared in this manner were more often unstable on storage than were those in which residual ether was evaporated in the cold.

The complement-fixing antigen appeared to be more stable when refrigerated in the crude formalinized suspension of tissue than it was in the ether-extracted stock preparation. Observations made on antigen 21, prepared from mouse lungs infected with the Imphal strain, will illustrate this point. The suspension was treated with formaldehyde on October 28, 1944, and a portion was extracted with ether on November 3. Its titer when determined the following day was ⅛ while on November 30 and December 23 it was ¼. Another portion of the crude antigen was extracted on December 21; its titer was 1/16 on December 23, ¼ on January 2, and ⅛ on January 25. A third portion was extracted on January 1st, and its titer was 1/16.

Several ether-extracted suspensions of formalinized tissue were dried from the frozen state. The complement-fixing titers of the rehydrated materials were considerably less than those of the original suspension.

The complement-fixing antigen of scrub typhus is relatively stable under certain conditions. Body fluids which contain it can be stored and dehydrated without affecting its serological activity. However, when present in treated suspensions of infected tissue the antigen is somewhat less resistant to storage and manipulation; furthermore, its behavior in different lots of material is variable. Blood and pleural fluid from mice and rats that die within 4 to 6 days after infection provide a readily available and useful source of scrub typhus antigen for serological work.

DISCUSSION

Complement-fixing antigen of scrub typhus occurs in demonstrable amounts in the body fluids and tissues of mice, white rats, and cotton rats in which a rapidly progressive, fatal disease is experimentally induced with the rick-

ettsiae. A simultaneous infection of myriads of host cells may be required in order to liberate enough of the specific substance at one time for it to be detected by serological methods. On the other hand, the appearance of specific antibodies in mice with a protracted disease may be responsible for the failure to detect scrub typhus antigen in materials from those animals that die after the first week. Probably both factors are of importance under the conditions of our experiments.

The complement-fixing antigen has a mass considerably less than that of the intact bacillary rickettsial structure. Ultracentrifugal studies indicate that the complement-fixing substance belongs in the class of soluble antigens. Whether the antigen is a homogeneous molecular substance such as the L-S protein of vaccinia (15) or whether it consists of inhomogeneous fragments of the rickettsial organism remains to be determined.

The amount of soluble antigen present in preparations of tissue infected with viruses or rickettsiae is usually proportional to the infective titer of the organ. This relationship is affected by a number of factors. For example, the *in vitro* techniques employed with soluble antigens are generally less sensitive than the *in vivo* methods used to determine infectivity. Therefore, a preparation may have a moderately high infectious titer yet contain no demonstrable serological activity. Furthermore, the infectivity of a given material is usually affected more drastically by manipulation in the laboratory than is the serologically active substance. The ordinary relation-ship between these two types of biological material appears to exist in tissues of rodents infected with scrub typhus but does not apply to the blood of these animals. This is to be expected since the tissues serve as a culture medium for the agent while blood behaves primarily as a diluent for the organism and its products. The proportionately greater amount of soluble antigen than of rickettsiae in blood may have resulted from a greater absorption of the soluble substance from diseased organs or from a greater destruction of the intact organisms than of the antigen in the circulating blood.

SUMMARY

A complement-fixing antigen specific for scrub typhus occurs in the body fluids and tissues of infected mice, white rats, and cotton rats.

The specific serological substance is demonstrable only in those animals which develop a rapidly fatal disease after an incubation period of a few days. Such an experimental infection is induced in mice and rats by the intravenous injection of suspensions of yolk sac rich in *R. orientalis*.

Ether extraction is an important step in the preparation of a complement-fixing antigen from tissues of mice dying with scrub typhus.

The Imphal No. 8 and Calcutta strains of *R. orientalis* are indistinguishable on the basis of complement fixation and cross-immunity tests.

The complement-fixing antigen in body fluids of infected mice and rats and in our preparations of tissues from such animals occurs as a soluble antigen. Under the proper conditions the soluble antigen can be stored or dehydrated without loss of serological activity.

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Addendum.—Since this article went to press two papers have appeared which should be mentioned.

(*a*) Fulton, F., and Joyner, A., *Lancet*, 1945, **2,** 729.

These authors found that suspensions of *R. orientalis* obtained by washing ultrasediment from fresh infectious suspensions of mouse lung tissue were usable as complement-fixing antigen. In view of the differences in techniques employed in the two laboratories, their failure to demonstrate soluble antigen in supernatant fluids (type not clearly indicated) does not conflict with our observations.

(*b*) Bengtson, I. A., *Pub. Health Rep., U.S.P.H.S.*, 1945, **60,** 1483.

The author demonstrated serological differences between the Karp and Gilliam strains of *R. orientalis* by means of the complement fixation technique.