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Identification of Rickettsial Agents Isolated in Guinea Pigs by Means of Specific Complement Fixation.

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The isolation of the agents of rickettsial diseases of man are usually made in guinea pigs (scrub typhus is an exception, since the white mouse is more susceptible). Once isolated the agent is identified by its capacity to induce a febrile and pathologic response in guinea pigs (scrotal swelling or necrosis, enlarged spleen, etc.) or the absence of these reactions in other species of animals, followed by the demonstration of a reciprocal cross immunity with known rickettsial strains. This procedure is not only time-consuming and expensive but presents certain difficulties because strains of the agent of a given disease may induce reactions in guinea pigs of different degree and intensity. The problem of strain identification, based on animal reactions alone, can therefore, present certain difficulties, some of which are discussed here.

When human blood, from a case of epidemic typhus, is inoculated intraperitoneally into male guinea pigs (400-500 g), the incubation period may vary from 6 to 20 days followed by a febrile period of variable duration. When brain suspensions from these animals are passaged to other male guinea pigs the incubation period is shortened to 6 or 7 days but may vary from 4 to 14 days. (Data based on 54 separate isolations).†

While scrotal swelling is not frequent in epidemic typhus, this reaction may occasionally occur. Since epidemic and murine typhus confer a reciprocal cross immunity in guinea pigs further identification of the agent is made in white rats, in which animal the epidemic disease cannot be maintained by serial passage, while this is possible with murine typhus. Since fever is an important expression of the experimental disease, the interpretation of a reaction can be confused when fever is caused by conditions other than typhus. Furthermore, there are a certain number of guinea pigs that develop an inapparent disease, which is characterized by the absence of a febrile reaction.

When infectious human blood from a case of murine typhus is inoculated intraperitoneally into male guinea pigs (400-500 g), the incubation period can vary from 4 to 14 days followed by a febrile period of variable duration. When scrotal exudate from these animals is passaged to other guinea pigs, the incubation period is usually shortened to about 4 or 5 days with a variation of from 2 to 10 days. This is then followed by a febrile period of variable duration.† While scrotal swelling is usually present in large guinea pigs and Neil-Mooser cells are readily demonstrated, this reaction may be absent in animals even though they develop a febrile reaction. There are other guinea pigs that develop an inapparent disease, with no febrile or scrotal reaction whatsoever.

The type of reaction induced in guinea pigs with the agent of Rocky Mountain spotted fever depends upon whether a mild or virulent strain is isolated. The period of incubation with the former is usually longer; furthermore, no scrotal necrosis occurs and the guinea pigs usually survive. With the virulent strain, the period of incubation is shorter, scrotal necrosis develops and death occurs in the majority of the animals inoculated. Identification of the spotted fever agent by cross immunity tests may cause some difficulty since a partial immunity to Rocky Mountain spotted fever is found in guinea pigs convalescent from typhus

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† Same technic used throughout. Details to be published.

TABLE I.
Murine Typhus.

Guinea pig No.	Incubation (days)	Fever (days)	Scrotal reaction	Complement fixation titer		
				Epidemic	Murine	Rocky Mountain spotted fever
3322	3	7	+	1/10	1/320	0
3328	3	7	+	1/20	1/160	0
3329	3	7	+	1/20	1/320	0
3325	4	6	+	1/10	1/160	0
3326	3	6	+	1/20	1/320	0
3330	3	6	0	1/20	1/320	0
3338	3	6	+	1/80	1/320	0
3347	4	6	0	1/80	1/640	0
3317	3	5	+	1/80	1/320	0
3319	3	5	+	0	1/160	0
3321	3	5	+	1/20	1/160	0
3331	3	5	+	1/10	1/160	0
3332	3	5	+	1/10	1/160	0
3334	3	5	+	1/20	1/320	0
3337	3	5	+	1/10	1/80	0
3341	4	5	0	1/40	1/320	0
3323	4	4	+	1/20	1/320	0
3335	5	4	+	1/80	1/640	0
3344	5	4	+	1/20	1/160	0
3350	3	4	+	1/10	1/160	0
3308	6	3	0	1/40	1/320	0
3311	3	3	0	1/10	1/320	0
3316	5	3	+	1/80	1/320	0
3318	4	3	+	0	1/160	0
3320	4	3	+	1/20	1/320	0
3324	4	3	+	1/20	1/320	0
3327	5	3	+	1/20	1/320	0
3349	5	3	+	0	1/160	0
3355	3	3	+	1/40	1/320	0
3307	5	2	0	1/10	1/160	0
3309	5	2	0	1/40	1/320	0
3313	4	2	+	1/40	1/640	0
3333	7	2	0	1/20	1/320	0
3339	4	2	0	1/20	1/320	0
3345	5	2	+	1/20	1/320	0
3352	5	2	+	1/80	1/640	0
3306	5	1	+	1/20	1/160	0
3314	6	1	+	1/40	1/320	0
3346	5	1	+	1/20	1/320	0
3354	5	1	+	1/20	1/320	0
3342	0	0	0	1/20	1/320	0
3348	0	0	0	1/10	1/160	0

fever and vice versa.[1,2]

During the past 3 years, this laboratory has employed the specific complement fixation test on specimens of convalescent guinea pig serum to differentiate epidemic and murine typhus and Rocky Mountain spotted fever. This paper will deal with the results obtained in an experimental study of infections in guinea pigs produced with strains of these 3 agents.

Experimental. Murine Typhus: A series of 50 male guinea pigs (400-500 g) was bled prior to inoculation in order to obtain baseline specimens. They were then inoculated intraperitoneally at the same time with the same pooled suspension of 1 cc of tunica vaginalis exudate—Wilmington strain. (Both tunica vaginalis and testicular exudate are suspended in 20 cc of physiological saline.) Forty-two of the 50 guinea pigs survived the entire experiment, the others dying of intercurrent infections.

Forty guinea pigs developed fever while 2

[1] Castaneda, M. R., and Silva, R., *J. Immunol.*, 1941, **42**, 1.

[2] Parker, R. R., *Public Health Rep.*, 1943, **58**, 721.

showed no febrile reaction. The period of incubation varied from 3 to 7 days and the febrile period from 1 to 7 days. Eleven or 26% of the 42 guinea pigs did not develop a scrotal reaction and 2 of these likewise did not develop fever.

About 4 weeks after the initial inoculation, all of the guinea pigs were bled and complement fixation tests for epidemic and murine typhus and Rocky Mountain spotted fever were performed, using the specific purified rickettsial antigens already described.[3,4,5,6] It had been shown that the soluble or "common" antigen gave cross fixation between epidemic and murine typhus and, hence, differentiation between these diseases could not be made when this material was present. Differentiation between these closely related diseases was possible, however, when washed rickettsial suspensions, free of soluble antigen, were used as antigen. Table I summarizes the results obtained.

All pre-inoculation specimens were negative in complement fixation tests with the epidemic, murine and Rocky Mountain spotted fever antigens. The convalescent specimens all gave positive reactions with the murine antigen; the titers varied between 1/80 to 1/640 with values of 1/320 or greater in more than half of the specimens. All but 3 of the sera also reacted with the epidemic antigen; nevertheless, the titers were always considerably lower than those obtained with the murine antigen. The serological response of guinea pigs 3307, 3309, 3311, 3330, 3333, 3339, 3341, 3342, 3347 and 3348, which developed a febrile reaction without scrotal swelling, was indistinguishable from that of guinea pigs which developed both fever and scrotal swelling. Guinea pigs 3342 and 3348 were of particular interest, for they had neither a febrile reaction nor scrotal swelling, yet they also developed complement fixing antibodies. This serological response, together with the subsequent demonstration of an immunity to re-infection, indicated that these 2 guinea pigs had had an inapparent infection. There appears to be no correlation between the titers obtained and the severity of the experimental infection, as judged by the duration of the febrile period or the presence or absence of scrotal swelling. All specimens of serum were negative in tests with the Rocky Mountain spotted fever antigen.

One month after the temperatures returned to normal the guinea pigs were challenged by the intraperitoneal inoculation of 1 cc of a tunica vaginalis exudate suspension (Wilmington strain). All of the guinea pigs proved immune, while the controls reacted in the usual manner.

Epidemic Typhus. Fifty male guinea pigs (400-500 g) were bled for determination of pre-inoculation antibody levels and then injected intraperitoneally at the same time with 1 cc of a 10% pooled brain suspension (Breinl strain—guinea pigs sacrificed on the third febrile day). Thirty-nine of the 50 guinea pigs survived the entire experiment, the others dying of intercurrent infection. Thirty-three guinea pigs developed fever while 6 showed no febrile reaction. The period of incubation varied from 5 to 19 days followed by a febrile period of from 1 to 10 days. Four of the guinea pigs developed scrotal swelling. About 4 weeks after the initial inoculation all of the guinea pigs were bled and complement fixation tests for epidemic and murine typhus and Rocky Mountain spotted fever were performed on samples of serum. Table II summarizes the results obtained.

All pre-inoculation specimens gave negative results in complement fixation tests with the epidemic, murine or Rocky Mountain spotted fever antigens. Sera from 32 of the 33 guinea pigs that developed fever showed complement fixation titers of 1/80 to 1/640, the majority being 1/320 or higher, with the epidemic antigen. When cross fixation occurred with the murine antigen, the titers in these 8 instances were low, never exceeding 1/40. None of the specimens reacted with the Rocky

[3] Plotz, H., and Wertman, K., *Science*, 1942, **95**, 441.

[4] Plotz, H., *Science*, 1943, **97**, 20.

[5] Plotz, H., Wertman, K., Bennett, B. L., Report to the Surgeon General, February 15, 1944, to be published.

[6] Plotz, H., Wertman, K., and Reagan, R. L., *Bulletin of the U. S. Army Medical Department*, 1944, **79**, 40.

TABLE II.
Epidemic Typhus.

Guinea pig No.	Incubation (days)	Fever (days)	Complement fixation titer		
			Epidemic	Murine	Rocky Mountain spotted fever
764	6	10	1/320	1/10	0
765	5	9	1/320	0	0
771	5	9	1/320	0	0
981	5	9	1/640	1/20	0
759	8	8*	1/160	0	0
761	6	8*	1/320	1/10	0
768	5	8	1/160	0	0
994	6	8	1/160	0	0
736	8	7	1/320	0	0
738	6	7*	1/640	1/40	0
758	7	7	1/320	0	0
767	9	7	1/160	0	0
769	5	7	1/320	0	0
740	6	6	1/160	0	0
775	7	6	1/640	0	0
979	6	6*	1/320	0	0
980	5	6	1/320	0	0
984	7	6	1/320	0	0
739	7	5	1/320	0	0
757	12	5	1/80	0	0
770	10	5	1/160	0	0
742	9	4	1/320	1/10	0
743	7	4	1/640	0	0
772	10	4	1/160	0	0
773	9	4	1/160	0	0
987	9	4	1/320	0	0
990	10	4	1/160	1/20	0
991	6	4	1/160	0	0
766	5	3	1/160	1/20	0
760	6	3	1/640	1/20	0
977	19	2	1/640	0	0
985	7	1	1/80	0	0
986	15	1	0	0	0
737	0	0	0	0	0
741	0	0	0	0	0
763	0	0	0	0	0
983	0	0	0	0	0
988	0	0	0	0	0
989	0	0	1/160	0	0

* Also had scrotal swelling.

Mountain spotted fever antigen. Guinea pig 986, that developed no complement fixing antibodies, had fever of 104.5° F. for one day's duration on the 15th day after inoculation. When subsequently challenged, with an epidemic strain, this animal responded with a febrile reaction; furthermore, the epidemic complement fixing antibodies then appeared in its serum. Thus the fever which was observed in this animal on the 15th day after primary inoculation was due to causes other than epidemic typhus. The other 31 members of the group were immune when challenged with epidemic typhus material.

There were 6 guinea pigs that did not develop a febrile reaction after the initial inoculation of infectious material (see No. 737, 741, 763, 983, 988, and 987 in Table II). It is observed that one of these guinea pigs, No. 989, developed specific epidemic complement fixing antibodies in convalescence. This animal proved to be immune to epidemic typhus when challenged subsequently. Therefore, guinea pig No. 989 had an inapparent infection as a result of the primary inoculation. In contrast, the other 5 guinea pigs in this group were not immune when challenged with an epidemic strain. It is considered

TABLE III.
Rocky Mountain Spotted Fever.

Guinea pig No.	Incubation (days)	Fever (days)	Complement fixation titer		
			Epidemic	Murine	Rocky Mountain spotted fever
294	3	9	0	0	1/320
280	3	8	0	0	1/320
323	2	8	0	0	1/320
290	3	7	0	0	1/640
292	4	7	0	0	1/320
276	3	7	0	0	1/640
260	4	7	0	0	1/320
286	4	7	0	0	1/320
288	4	7	0	0	1/320
190	6	7	0	0	1/320
191	2	6	0	0	1/320
284	5	5	0	0	1/320
189	6	2	0	0	1/640

that these animals represent "misses" in that the inoculated infectious material induced neither fever, an antibody response nor immunity.

In another series of 50 guinea pigs infected with different strains of epidemic typhus, 2 guinea pigs were observed which developed no febrile reaction. Convalescent specimens of serum from both of these guinea pigs showed the presence of epidemic complement fixing antibodies and the animals proved immune on challenge. The other 48 animals in this series displayed fever after primary inoculation, developed complement fixing antibodies, and were subsequently immune when challenged. Further evidence was acquired on the development of complement fixing antibodies in guinea pigs with an inapparent infection when 30 animals were inoculated with small doses of epidemic typhus rickettsiae. Seventeen of the animals responded in the usual manner. Thirteen individuals in the group developed no febrile reaction after inoculation but epidemic complement fixing antibodies were demonstrated in samples of serum obtained one month later from each of the thirteen. Finally, each of these animals proved to be immune when challenged with epidemic typhus.

Rocky Mountain Spotted Fever. Thirteen guinea pigs were bled for serological studies and then inoculated intraperitoneally with 1 cc amounts of a pool of infectious blood obtained from guinea pigs acutely ill with a mild strain of Rocky Mountain spotted fever. A febrile period of from 2 to 9 days followed an incubation period varying from 2 to 6 days. The guinea pigs were bled 14 days after the temperature returned to normal. Serum from each animal contained complement fixing antibodies that reacted with the Rocky Mountain spotted fever antigen and none that reacted with the epidemic or murine antigen. All preinoculation specimens were negative with the 3 antigens. Data from this experiment are summarized in Table III.

Discussion. The identification of strains of epidemic and murine typhus or Rocky Mountain spotted fever based on animal reactions alone presents certain experimental difficulties. This has been illustrated when guinea pigs were inoculated at the same time with the same amount of a pool of infectious material. Irrespective as to whether the guinea pig develops evidence of disease as expressed by a febrile reaction or scrotal swelling, or an inapparent disease without these reactions, specific complement fixing antibodies develop in early convalescence. The use of the complement fixation reaction, likewise, permits the detection of those animals that represent missed infections or those that develop fever from non-specific causes. The use of the complement fixation method for strain identification is specific, rapid and inexpensive.

Summary. 1. Guinea pigs inoculated intraperitoneally at the same time with the

same amount of a pooled infectious suspension of tunica vaginalis washings, Wilmington strain of murine typhus, developed three types of reaction following a variable period of incubation: (a) fever of variable duration and scrotal swelling, followed by the appearance of specific murine complement fixing antibodies; (b) fever of variable duration and no scrotal swelling followed by the appearance of specific murine complement fixing antibodies; and (c) no febrile reaction or scrotal swelling but followed by the appearance of specific murine complement fixing antibodies (inapparent infection). All guinea pigs showing one of these three types of response were proven immune when challenged subsequently.

2. Guinea pigs inoculated intraperitoneally at the same time with the same amount of pooled infectious brain suspension, Breinl strain of epidemic typhus, developed 3 types of reaction following a variable period of incubation: (a) febrile period of variable duration followed by the appearance of specific epidemic complement fixing antibodies; (b) no febrile reaction but followed by the appearance of specific epidemic complement fixing antibodies (inapparent infection); and (c) no febrile reaction followed by no complement fixing antibodies. This latter group are regarded as "misses" for these guinea pigs were not immune when challenged while those of group a and b were immune.

3. Specific murine or epidemic complement fixing antibodies were demonstrated after the inapparent infections with these 2 types of typhus.

4. Guinea pigs inoculated at the same time with the same amount of pooled infectious blood of Rocky Mountain spotted fever (mild strain) exhibit a febrile reaction of variable duration following a variable period of incubation. In all instances specific complement fixing antibodies to Rocky Mountain spotted fever were demonstrated in convalescent specimens of serum and no complement fixing antibodies with a murine or epidemic antigen.

Conclusion. The specific complement fixation test, using purified rickettsial suspensions as antigen, is recommended as a routine test to differentiate infections in guinea pigs caused by rickettsiae of murine and epidemic typhus and Rocky Mountain spotted fever.

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Studies on Chancroid. IV. The Ducrey Bacillus: Growth Requirements and Inhibition by Antibiotic Agents.*

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The classification of the Ducrey bacillus in the genus *Hemophilus* appears to be based largely on its morphology and on the fact that it grows best in a medium containing blood. Actually the growth requirements and biology of this organism have received very little study. The present article is a report of some observations made in the course of a clinical and laboratory investigation of chancroid. The data presented support the view that there are important differences between the Ducrey bacillus and other members of the genus *Hemophilus*.

Materials and Methods. The base medium used was proteose peptone broth (Difco). Blood was obtained from rabbits by cardiac puncture. Defibrination was accomplished by shaking with glass beads. Erythrocyte suspensions were prepared by washing repeatedly with large volumes of normal saline, until a negative Pandy test was obtained on the supernatant fluid.

X factor was prepared as follows: The clot

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