DEMONSTRATION OF AGGLUTININS FOR BARTONELLA BACILLIFORMIS

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Human bartonellosis, known as Carrion's disease, manifests itself in two distinct phases. The first phase, Oroya fever, is a severe hemolytic anemia, the result of the invasion of the blood stream by the organism, *Bartonella bacilliformis*. Blood cultures are strongly positive at this stage of the disease; and the red blood cells, as well as the cells of the reticulo-endothelial system, are heavily parasitized by *Bartonella*. The second phase, veurrga peruana, is characterized by the appearance of the typical cutaneous eruption, from the nodules of which *B. bacilliformis* may be recovered. Bartonellosis is endemic in certain regions of Peru, and has more recently been found to be so in Colombia and Ecuador. In these endemic regions, the second stage (verruga) is more commonly encountered. Less frequently, the anemic stage, when very severe, ends fatally before the development of the eruption. A large percentage of the native population of the endemic zones in Peru have had some form of bartonellosis, mild or severe, and are able to live there without further clinical evidence of infection. Second attacks of acute fever without the eruption are apparently unusual; but second attacks of verruga with minimal febrile illness are not rare. From these facts, it is evident that in most of those individuals who have contracted bartonellosis at least once, a definite and long lasting immunity develops against the infection. This immunity on the part of the natives is in strong contrast to the often fatal infection which develops in individuals from non-endemic zones who spend but a few nights in the endemic zones without adequate protection against the night flying sandfly vector. Somewhere in between these two extremes of complete immunity on the one hand, and complete susceptibility on the other, if one

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speculates in terms of immunology, must lie the so called carriers, thought by Weinman and Pinkerton (1) to represent a possible reservoir of infection. These authors have estimated the number of such individuals in one locality of the endemic region of Peru at about 5 to 10 per cent of the total population. They show no outward sign of infection; but *B. bacilliformis* may be recovered from their blood, which may or may not show a slight anemia. It is evident from the foregoing rather empirical facts that the fundamental immunological pattern of human bartonellosis remains still to be worked out. The present report is a step toward the solution of this complex problem.

The work which follows deals with the production and the demonstration of specific agglutinins for *B. bacilliformis* in rabbits, and their demonstration in the sera of human beings with bartonellosis. In the literature on Carrion's disease, the possibility of agglutinating *B. bacilliformis* with specific sera of one sort or another has been considered from time to time by various authors (2–7). In no instance is there any indication of clear-cut experiments on this aspect of the problem; and in all cases the only tangible conclusion that has been reached is that any demonstration of agglutinins is handicapped by the difficulties encountered in preparing suspensions of the organism suitable for use as an antigen.

Materials and Methods

Media and Cultivation of Bartonella.—Heretofore, the only media that have yielded fairly satisfactory growth have been ordinary blood agar and the semisolid medium of Noguchi and Battistini (3) originally designed for the cultivation of *Leptospira*. The former medium yields colonies of tightly packed organisms on the slant, with many of the colonies embedded in agar; the latter medium a variable and diffuse growth, with aggregates of smaller satellite granules, in the upper 1 to 2 cm. of the medium. Both types of medium present obstacles to the preparation of a homogeneous suspension, chief among which is that resulting from the presence of agar, from which the organisms are inseparable. Two types of medium recently developed by Geiman in Boston (8) have effectively removed these obstacles.

The first is a solid medium. Special base agar is made with 2 per cent shredded agar, 2 per cent tryptone or proteose peptone, 0.5 per cent sodium chloride, in distilled water. The medium, as finally used, is composed of 75 per cent base agar, adjusted to pH 7.6–7.8 and cooled to 45°C.; and 25 per cent fresh rabbit or sheep serum, or defibrinated whole blood. To the whole medium, a small amount (0.2 per cent of the total volume of medium) of an ascorbic acid-glutathione solution is added. This medium gives a profuse growth, which follows the streak of the loop on the slant and becomes grossly visible in 24 to 48 hours, reaching a maximum in 10 to 14 days. The optimal temperature for incubation is 28°C. The growth may be easily scraped from the surface, and collected from the water of condensation at the bottom of the tube.

The second is a liquid medium. It is composed of three parts of a 1 per cent solution of tryptone adjusted to pH 7.6–7.8, to one part fresh rabbit serum; and to the whole medium is added a small amount (0.2 per cent of the total volume of medium) of the ascorbic acid-glutathione solution. Growth becomes visible as a finely divided sediment within 24 to 48 hours, and reaches a peak on the 10th day, when incubated at 28°C. It has been found by Geiman that maximum growth takes place at the bottom of the container when the depth of this medium is from 0.5 to 0.75 cm. Small (50 cc.) Erlenmeyer flasks are used, to afford as large an area as possible, at this optimal depth, for the growth which takes place on the bottom of the flask. The present writer has found that ordinary test tubes (14 cm. × 2 cm.), bent to resemble a hockey stick in shape, with the short arm at the open end of the tube, serve better than Erlenmeyer flasks for the cultivation of *Bartonella*. The tubes are stored horizontally, the open end directed upward, to prevent outflow of the medium with which the tubes are filled to half of their horizontal depth, *i.e.* 0.5 to 0.75 cm. Growth takes place along the bottom of the converted test tube, and the yield is about equal to that obtained with the flasks. There is further the added advantage of convenience; and less danger of contamination than that encountered with the flasks.

Preparation of Antigen.—The organisms harvested from both types of medium, when washed two or three times in normal saline, and resuspended in saline buffered to pH 8.2–8.4, constitute a homogeneous antigen admirably suited for agglutination tests. It has been found more recently that clumps of organisms harvested in a similar manner, but stored as a coarse suspension in normal unbuffered saline can, after about a week of such storage, be easily emulsified by churning with a capillary pipette, (9). The coarser particles in either type of suspension are allowed to settle or are thrown down by slow centrifugation. In both cases, dark field examination reveals a fairly regular distribution of single organisms, with occasional clumps of never more than 5 to 10 (Fig. 1). These occasional clumps are just visible with a 10 or 14 power hand lens.

Technique of Agglutination Test.—The technique of the test for agglutinins has been designed to allow rigid economy of materials, and at the same time permit accurate gradation of titre. Small fermentation tubes (7.5 cm. × 0.75 cm.) are used; and the total final volume confined to about 0.1 cc. The tubes, after proper serial dilution of the sera and the addition of the antigen, are incubated for 4 to 6 hours at 40°C. and are then read with the aid of a 10–14 × hand lens; and after 24 hours on ice they are again read. In the strongly positive tubes, there is a heavy and tightly coherent precipitate with clear supernatant liquid. Dark field examination (Fig. 2) reveals a definite heavy clumping of the single organisms and the small groups of organisms seen in the control (Fig. 1). In the negative and the control tubes, when examined with the hand lens, there is never more than a very slight sediment, which is easily dispersed.

Immunization of Rabbits.—To obtain specific agglutinins, a series of rabbits was immunized by repeated injections of living *B. bacilliformis* into the marginal vein of the ear. The organisms from a tube of liquid culture, after 10 days to 2 weeks incubation, or a week's growth from a solid slant, washed twice with saline, was given every 5th day over a period of 60 days, each animal having received 12 to 13 inocula-

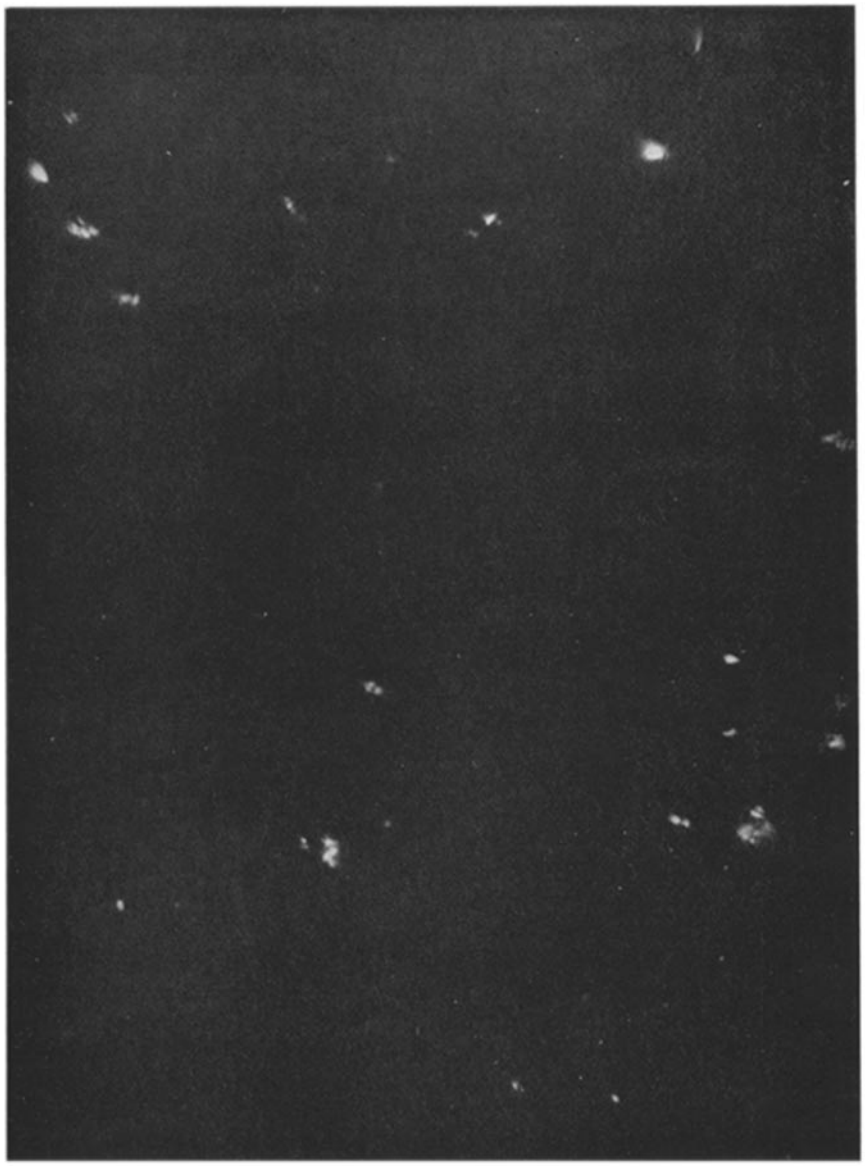

FIG. 1. Suspension of *Bartonella bacilliformis* in normal saline. Dark field photomicrograph. × about 4000, oil immersion.

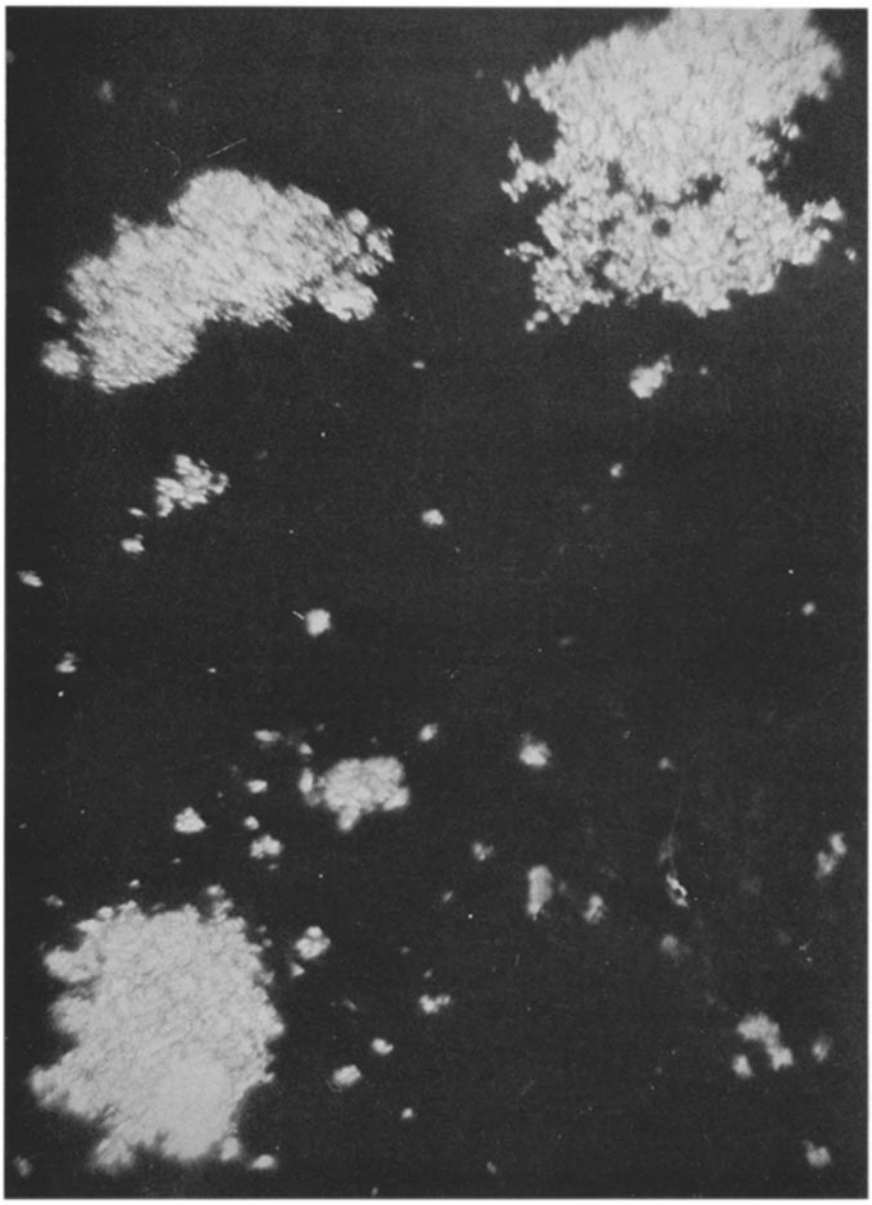

FIG. 2. Agglutination of *Bartonella bacilliformis* by serum of immunized rabbit 1 in dilution of 1:640. Dark field photomicrograph. × about 4000, oil immersion.

tions in all at the end of that time. Two rabbits were treated in a manner different from the others. One of these (rabbit 1) was inoculated every 3rd or 4th day with the material and by the method described above, 15 inoculations having been given at the end of 50 days. The other rabbit (rabbit 2) had been splenectomized, and into the anterior chamber of both eyes, chopped rabbit embryo had been successfully implanted. *B. bacilliformis* was then injected into both anterior chambers, and given in large quantity intratracheally, in an attempt to produce some tangible evidence of pathogenic effect. 2 weeks after the intratracheal and intraocular inoculations, one eye, which was then aseptically removed, showed a marked pannus formation, with ingrowth of vessels into the cornea; and the other eye showed nothing. Cultures taken from the implanted tissue and the aqueous humor of the enucleated eye showed a heavy growth of *B. bacilliformis*; but blood cultures were negative. Serum for agglutination was taken at this time. At no time did any of the animals, no matter in what way they had been inoculated, show any signs of systemic reaction which could have been interpreted as having resulted from the inoculation.

Results with Rabbit Sera

The results obtained in testing the sera of these rabbits for agglutinins by the method described are shown in Table I. In the case of rabbits 1 and 3, in order to determine the rate at which antibodies might be appearing, serum was taken after one-half the total projected number of inoculations had been given; and showed a moderate titre of agglutinins. The titre as determined after all inoculations had been given was proportionately higher in all cases. The definitely higher titre of rabbit 1 as compared with that of rabbits 3, 4, and 5 might be explained by the larger number of inoculations given at shorter intervals to rabbit 1 (15 inoculations in 50 days as opposed to 12 in 60 days); although this difference in titre may only be an indication of wide variation in the strength of response among individual animals. There was in the case of rabbits 1, 3, and 4 a marked falling-off of titre at the end of 1 month after the last inoculation. Rabbit 5 showed a somewhat higher titre of agglutinins than did the other two, and maintained this higher titre longer than the latter, although there was a slight but definite falling-off at the end of 1 month after the last inoculation. In the case of rabbit 2 the titre of agglutinins obtained as a result of the simultaneous intraocular and intratracheal inoculations described above, lies midway between that of rabbit 1 and those of rabbits 3 and 4. There is no indication, in this single experiment (rabbit 2), as to which method of inoculation may have resulted in so high a titre, or whether both were necessary to produce it. The fact that cultures made from the eye fluid and the infected implanted embryonic tissue were strongly positive after 2 weeks suggests the possibility that these two foci were a source of continuous stimulation to the production of antibodies by the host.

Results with Human Sera

Table II summarizes the findings with a small group of human sera. Two cases in which the anemic manifestation had been minimal and the verrucous

TABLE I

Agglutination of Bartonella bacilliformis by Serum of Immunized Rabbits

Rabbit	Time	Final dilution of serum						
		1:10	1:20	1:40	1:80	1:160	1:320	1:640
1	Before immunization	−	−	−	−	−	−	−
	After 7 intravenous inoculations in 22 days	4+	4+	4+	4+	2+	−	−
	5 days after last of 15 intravenous inoculations given over 50 days	4+	4+	4+	4+	4+	4+	3+
	28 days after last intravenous inoculation	4+	4+	4+	3+	2+	−	−
2	Before intratracheal and intraocular inoculation	−	−	−	−	−	−	−
	13 days after intratracheal and intraocular inoculation	4+	4+	3+	3+	+.	−	−
	34 days after intratracheal and intraocular inoculation	4+	4+	4+	4+	3+	3+	−
3	Before immunization	−	−	−	−	−	−	
	After 7 intravenous inoculations in 33 days	2+	2+	2+	+	+	−	
	3 days after last of 13 intravenous inoculations given over 60 days	4+	4+	4+	3+	2+	−	
	36 days after last intravenous inoculation	4+	4+	4+	+	−	−	
4	Before immunization	−	−	−	−	−	−	
	5 days after last of 12 intravenous inoculations given over 60 days	4+	4+	3+	2+	2+	+	
	37 days after last intravenous inoculation	3+	3+	3+	+	−	−	
5	Before immunization	−	−	−	−	−	−	
	3 days after last of 13 intravenous inoculations given over 64 days	3+	3+	3+	3+	2+	2+	
	36 days after last intravenous inoculation	3+	3+	3+	2+	2+	2+	

4+, heavy coarse precipitate at bottom of tube; clear supernatant liquid.

3+ to +, gradually decreasing amounts of precipitate, but obvious agglutination of suspended organisms when compared with saline and normal serum controls.

−, no agglutination.

stage more extensive (cases 1 and 5) showed the highest titre of agglutinins; and the two severe cases of Oroya fever (cases 2 and 3) the lowest. Thirteen normal human sera were consistently and entirely negative. Although definite

conclusions cannot be drawn from so small a series of cases, the possibility that the appearance of the eruption is associated with the formation of a demonstrable titre of circulating antibodies may be considered. The development of the eruption is invariably regarded by experienced clinicians as a favorable prognostic sign. The fact that the serum of the so called "immune" (case 6) contained a minimal titre of agglutinins may be analogous to the falling-off of titre noted with the rabbit sera. A satisfactory test for the virulence of the organism concerned (and hence a susceptible laboratory animal) would, of course, be necessary in the final elucidation of the fundamental immunological process involved in human bartonellosis.

TABLE II

Agglutination of Bartonella bacilliformis with Sera of Patients Suffering or Recovered from Bartonellosis (Carrion's Disease)

Case	Final dilution of serum			
	1:10	1:20	1:40	1:80
1	2+	+	+	±
2	+	+	−	−
3	+	±	−	−
4	+	−	−	−
5	2+	+	+	±
6	+	±	−	−
Normal human serum*.....	−	−	−	−

* Thirteen normal human sera were tested.

4+, heavy coarse precipitate at bottom of tube; clear supernatant liquid.

3+ to +, decreasing amounts of precipitate at bottom of tube; but obvious agglutination of suspended organisms when compared with saline and normal serum controls.

±, slight but definite agglutination, with no heavy precipitate.

−, no precipitate or agglutination.

The question of the relationship of *Bartonella* to the organisms of the *Rickettsia* group has also been explored. With this possible connection in view, the six human sera tested for agglutinins for *Bartonella* were also tested with three strains of *Proteus*, after the manner of the Weil-Felix reaction. The results are shown in Table III. The two sera which agglutinated *Bartonella* most strongly also show the highest titres with two of the *Proteus* strains (sera of cases 1 and 5). Although a positive agglutination at a dilution higher than 1:50 is usually considered significant with the Weil-Felix reaction, there are occasional cases without a positive history of typhus whose sera are positive in much higher dilutions. Since, also, the histories of the cases involved herein had not been probed for the possibility of typhus,

no definite conclusions can be drawn as to the meaning of the results obtained with these particular sera. The question becomes even more obscure from lack of knowledge of the distribution of endemic typhus fever in Peru. The only available figure is that of the total number of cases during the year

TABLE III

Agglutination of Proteus OX19, OXK, and OX2

Case	Strain	Final dilution of serum					
		1:8	1:16	1:32	1:64	1:128	1:256
1	Proteus OX19	4+	4+	4+	3+	2+	+
	Proteus OXK	3+	2+	2+	−	−	−
	Proteus OX2	4+	4+	2+	+	−	−
2	Proteus OX19	3+	2+	2+	+	−	−
	Proteus OXK	2+	−	−	−	−	−
	Proteus OX2	3+	2+	+	+	−	−
3	Proteus OX19	4+	3+	2+	+	−	−
	Proteus OXK	3+	2+	+	−	−	−
	Proteus OX2	4+	3+	+	−	−	−
4	Proteus OX19	2+	+	−	−	−	−
	Proteus OXK	4+	3+	+	−	−	−
	Proteus OX2	2+	−	−	−	−	−
5	Proteus OX19	4+	4+	3+	3+	+	−
	Proteus OXK	4+	4+	2+	+	−	−
	Proteus OX2	4+	4+	3+	2+	+	−
6	Proteus OX19	−	−	−	−	−	−
	Proteus OXK	3+	2+	2+	−	−	−
	Proteus OX2	+	−	−	−	−	−
Normal human serum	Proteus OX19	3+	+	−	−	−	−
	Proteus OXK	3+	2+	−	−	−	−
	Proteus OX2	3+	2+	+	−	−	−

All saline controls negative. *Bartonella*-immune rabbit serum negative with all strains of *Proteus*.

Tubes incubated 4 hours at 40°C.

Legend the same as that for Tables I and II.

1940, January through September, amounting to 667, no geographical allocation being given in the report (10). It is of importance to note, from an immunological point of view, that there was no agglutination of *Proteus* in significant titre by *Bartonella*-immune rabbit serum.

Origin of the Human Sera Represented in Tables II and III, with a Brief Description of Each Case

Case 1.—Serum was taken during the 3rd month of a third mild attack of bartonellosis, at which time there was slight anemia, and extensive eruption. Blood cultures were negative, having been positive during the 1st month of the recurring disease, during which time there had been no eruption.

Case 2.—Serum was taken on the 12th day of the disease. There was severe anemia (red blood cell count 1.7 million); blood cultures were positive; and *Bartonella* were found in the blood film. Death occurred on the 15th day of the disease.

Case 3.—Serum was taken on the 16th day of the disease. There was severe anemia (red blood cell count 1.0 million); blood cultures were positive; and *Bartonella* were found in the blood film. There was subsequent recovery, no *Bartonella* having been found in the blood films after the 26th day, at which time blood cultures also became negative.

Case 4.—Serum was taken during the 2nd month of a mild attack of verruga peruana, the eruption having appeared after 1 month of symptoms. Laboratory data were not available, and the history not precisely known. The eruption, however, was definitely that of verruga, but was confined to one large "mulaire" type of lesion on the left shin.

Case 5.—Serum was taken during the 2nd month of an attack of verruga peruana. The appearance of the rash was preceded by symptoms of malaise and joint pains. Laboratory data were not available, and the history not precisely known. The eruption, however, was definitely that of verruga, and was far more extensive than that of case 4.

Case 6.—This was a so called "immune," who, having had verruga in childhood, has remained free from signs and symptoms of bartonellosis all of his life (30-odd years) in spite of frequent and prolonged sojourns in the areas where Oroya fever and verruga peruana are endemic.

DISCUSSION

The fact that it is possible to produce circulating antibodies in rabbits, and that circulating antibodies may be demonstrated in human beings with bartonellosis makes it certain that at some stage during the course of an infection with *B. bacilliformis* there is a measurable immunological response on the part of the host. This response, now proven by the formation of agglutinins, can be used as a basis for further investigation with three principal objectives: 1) A clarification of the fundamental immunological sequence in human bartonellosis; 2) an evaluation of the properties of immune sera in the therapy of Oroya fever and of the effectiveness of vaccines in the prophylaxis of non-immune individuals; and 3) an elucidation of the antigenic and immunological characteristics of *B. bacilliformis*. With regard to the latter problem, preliminary studies have already been undertaken to determine possible immunological variations among *Bartonella* isolated from different sources and localities. These experiments have indicated that no immunological differ-

ences between such strains can be demonstrated by the agglutination test, cross-agglutination between four Peruvian and two Colombian strains by homologous and heterologous sera to almost equal titre having been accomplished.

SUMMARY

1. Methods of preparing a satisfactory antigen having been developed, a technique for performing an agglutination test with *B. bacilliformis* is made available.

2. As a result of repeated intravenous injection of living cultures of *B. bacilliformis* at short intervals, rabbits have been found to produce a high titre of specific agglutinins which, under the conditions obtaining in the present series of experiments, begins to decline after about one month following the last inoculation.

3. Sera from six cases of bartonellosis in different stages of its several manifestations have been shown by the agglutination test to contain a low but definite titre of circulating antibody.

4. Several of these same sera have been shown to contain as well a significantly high titre of agglutinins for three strains of *Proteus*. No definite conclusions can be drawn from this phenomenon since the case histories had not been probed for the possibility of typhus fever; and since the relatively high titres obtained with a few of the present sera may very well fall within the extremes of normal serum titres.

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