THE ETIOLOGY OF BARTONELLA MURIS ANEMIA OF THE ALBINO RAT

THE ISOLATION OF BARTONELLA MURIS

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PLATE 36

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As the present paper will show, it has been possible to isolate an organism in pure culture from the blood of splenectomized rats suffering from *Bartonella muris* anemia. A severe anemia was produced by the injection of this organism into 3 week old rats, rabbits, guinea pigs and young mice, all with intact spleens. The organism when injected into adult splenectomized Wistar Institute rats of non-carrier stock produced a mild anemia. During the height of the anemia occasional *Bartonella* bodies were found on the red cells. The strain of *Bartonella muris* was recovered in pure culture from these animals. The blood of the 3 week old rats and 3 week old rabbits, when injected into other immature animals of the same species, produced a transmissible infectious anemia.

Noguchi (1) isolated two organisms from the blood of a splenectomized rat, both of which he believed resembled *Bartonella muris* in morphology. The first grew on leptospira medium, but did not grow on ordinary culture media. This was a diphtheroid and was non-pathogenic for normal rats. From the blood of the same animal another minute Gram-negative non-motile bacterium was isolated on a blood plate. This organism grew on blood agar, was hemolytic but did not produce acid in sugars. It caused an acute orchitis in normal rats when injected intratesticularly.

Battistini and Weiss (2) report the isolation of an organism of the Salmonella group from the blood of splenectomized wild rats of Lima. They mention no experimental data supporting their contention of an etiological relationship with *Bartonella muris* anemia. They stress the similarity of the human Oroya fever to *Bartonella muris* anemia of the rat, and report unsuccessful attempts to transmit the rat anemia to monkeys, mice or guinea pigs with whole blood injections of anemic rats.

Lwoff and Vaucel (3) injected the blood of a dog which had been infected with *T. cruzi* (from a rat) into mice with intact spleens. *Bartonella* bodies appeared on the red cells in the mice. The *Bartonella* infection was probably carried from the rat through the dog. The *Bartonella* infection was transferred from mouse to mouse by injections of whole blood for 28 passages. These investigators isolated an organism from one of these mice on Noguchi's leptospira medium at 22–37°C. in 14 days and in N. N. N. medium (Nicolle, Novy, McNeal) in 10 days. The bacteria were both motile and non-motile, retained motility for 10 days and resembled morphologically *Bartonella bacilliformis*. In a mouse injected with 6 drops of the culture *Bartonella* bodies were occasionally seen on the 5th and 7th days. 26 days later the mouse was injected with the blood of a mouse heavily infected with *Bartonella muris* and developed a severe infection. Two other mice were injected with the culture. One remained free of infection and the other showed occasional *Bartonella* bodies on the red cells on the 4th to 7th day. The strain was carried through 3 subcultures in artificial media. It produced only a feeble infection and afforded no protection against subsequent infection.

Methods

The method of cultivation of *Bartonella muris* was the same as that employed by Noguchi for the isolation of *Bartonella bacilliformis*. The blood was withdrawn from the heart of an adult splenectomized rat at the height of the anemia in an equal volume of sterile isotonic sodium citrate solution. Dilutions of 1/10, 1/100, 1/1000, 1/10,000 and 1/100,000 were made of this blood with citrate. 0.4 cc. of each dilution was inoculated into tubes of Noguchi's leptospira medium at a pH of 7.4 and incubated at a temperature of 25°. Successful results were obtained in only two of many instances attempted. Growth appeared within 10 to 12 days as a fine cloud at the upper layer of the medium. By heavy seeding (0.2 cc.) the culture was transplanted to tubes of leptospira medium and after 2 transfers could be grown on blood agar slants.

Morphology

In the original culture there were scattered bacilli and coccobacilli with bent forms. They are fine rods varying from 0.4 to 3.2 micra in length and 0.2 to 0.4 micron in width with a predominance of short forms. On solid media the bacillary forms predominate and tend to clump with occasional thread formation. The sides of the bacilli are straight and the ends rounded. They are Gram-negative and very actively motile[1] on both the solid and semisolid media. When retransplanted from the solid to the semisolid medium the shorter pleo-

[1] After 4 months on artificial media the motility of the organisms markedly diminished.

morphic forms are predominant. After repeated subculture and animal passage the organisms are apt to be somewhat plumper than in the original cultures. Flagella are present.

Cultural Characteristics

Before animal passage the organism grew only in the presence of rabbit, horse or human blood which was added to hormone agar. This original strain has been grown on artificial media for 3 months and is still under observation.

On blood agar minute colonies appear in 48 hours. These are at first barely visible, translucent and round. They gradually increase in size and in a few days coalesce, forming a thin filmy tenacious growth on the surface of the medium. The color of older cultures is grayish with a tinge of yellow. The blood in the medium is not hemolyzed. 10 per cent solutions of each of sixteen sugars were added to Hiss serum water containing 0.2 per cent hemoglobin solution. On the sugar media containing glucose, maltose, saccharose, mannite, lactose, mannose, xylose, arabinose, raffinose, galactose, dextrin, levulose, salicin, inosite, inulin or dulcite, neither gas nor acid was produced during a period of 10 days. Litmus milk is slightly coagulated in 48 hours but no acid is formed. After animal passage the organism grew on glucose bouillon and ascitic agar without blood. In glucose bouillon the growth is limited to the upper layer. The organisms are actively motile on liquid, semisolid and solid media.

Cultures on solid media have a sweet odor, resembling canned pineapple. Old cultures, particularly on blood, have a faint herring odor. In liquid media after 10 days to 2 weeks a faint green pigmentation of the medium occurs. This pigment is insoluble in chloroform.

The optimal temperature for growth is 25°C., though slight growth occurs at 37°C. At room temperature the cultures in leptospira medium retain motility and viability for 38 days as determined by subculture.

Bartonella muris is differentiated from other Gram-negative motile bacilli by its cultural and biological characteristics.

Infection of 3 Week Old, 30 Gm. Rats with Bartonella muris Cultures

It has been demonstrated by Ford (4) and confirmed by the authors (5) that 3 week old, 30 gm. rats and rabbits with intact spleen will develop an anemia if injected with the blood of an anemic splenectomized rat.

Fourteen 3 week old, 30 gm. rats received 0.5 cc. of a 48 hour growth on leptospira medium or washings from young cultures on blood agar slants, intraperi-

toneally. Within 24 hours the rats became severely anemic, the hemoglobin dropped to below 40 per cent and in many instances the red cells fell to below 2,500,000 per c.mm. The anemia in the rats infected with the original culture was not as severe as that in the rats infected with subsequent subcultures. In both cases the anemia continued from 3 to 5 days after which the animals recovered. *Bartonella* bodies were found occasionally on the red cells. They were never numerous. Blood cultures made 48 hours after the onset of the infection were positive for *Bartonella muris*. Occasionally cultures of the liver and spleen were

TABLE I

3 week old rats injected with 0.5 cc. culture of *Bartonella muris* (Strain I-4°). (Sample protocol.)

Rat	Date		Red cell count	Hb (Dare)	*Bartonella* bodies	Blood culture
	1932			*per cent*		
K_1	Mar. 29	Before injection	5,500,000	110		
	" 30	After injection	3,200,000	40	Occasional *B. muris* bodies	
	" 31	" "	3,000,000	38	" "	Positive blood culture
	Apr. 1	" "				
	" 3					
	" 4					
K_2	Mar. 29	Before injection	4,900,000	92		
	" 30	After injection	2,600,000	45	Occasional *B. muris* bodies	
	" 31	" "	3,200,000	48		
	Apr. 1	" "	5,000,000	70		
	" 3	" "	4,800,000	75		
	" 4	" "	5,100,000	85		

positive. The organism was reisolated from the blood of the animals 1 to 5 days after injection. Colonies appeared within 48 hours after inoculation of the medium, at 25°C.

The culture reisolated from infected animals was pathogenic for other young rats in the same manner as the original culture.

The blood of the infected rats was infectious for other 30 gm. rats and the anemia produced was transmitted to other animals in series by injection of whole blood.

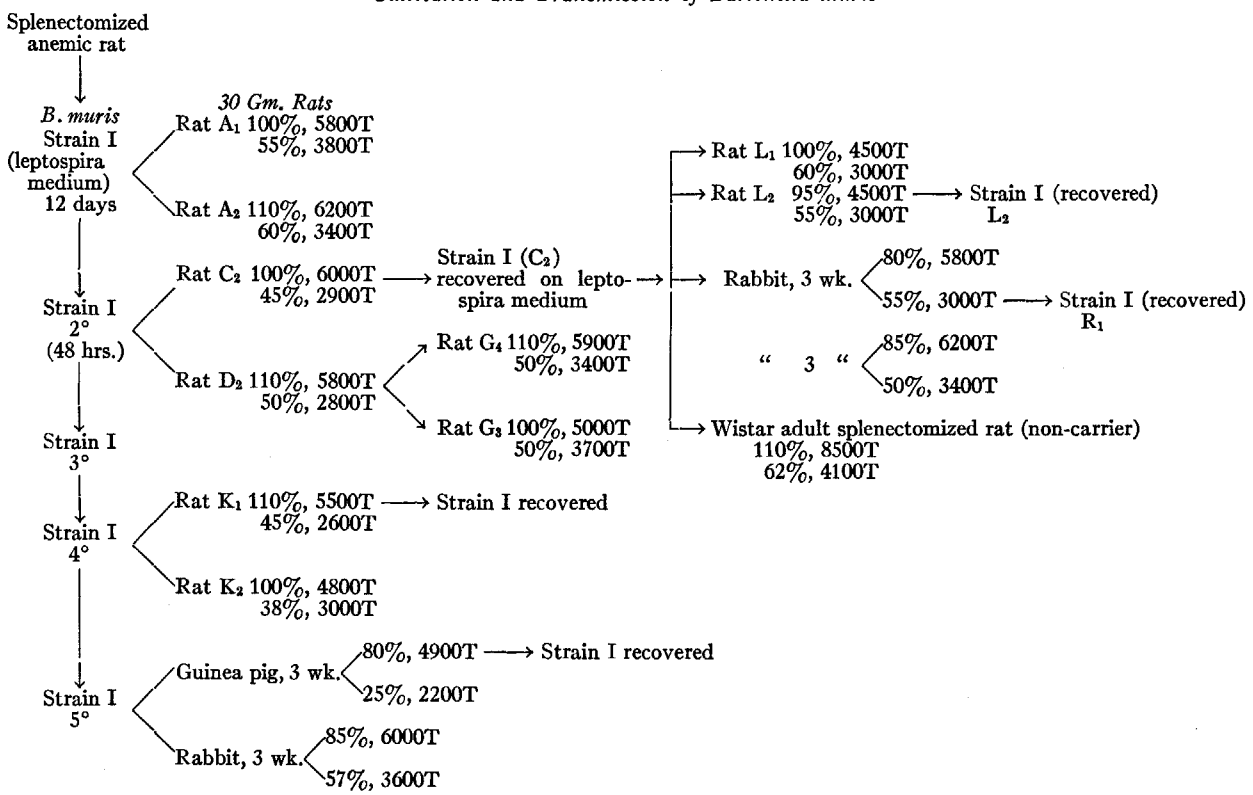

DIAGRAM 1

Infection of Suckling Rabbits with Bartonella muris Cultures

The susceptibility of the 3 week old rabbits with intact spleens (suckling) to *Bartonella muris* infection by the injection of whole blood of an anemic splenectomized rat as demonstrated by Ford (4) was likewise utilized to test the pathogenicity of the organism isolated from the anemic rat (*Bartonella muris*).

Three 3 week old rabbits received intravenously 0.5 and 1 cc. of a young culture of *Bartonella muris* on leptospira medium. A moderate anemia developed within 2 to 3 days, and reached its height on the 5th day after injection. The hemoglo-

TABLE II

3 week old rabbit injected with 1 cc. culture *Bartonella muris* (24 hour slant) intravenously. (Sample protocol.)

Rabbit No.	Date		Red cell count	Hb (Dare)	*Bartonella* bodies	Blood culture
	1932			*per cent*		
4	Apr. 8	Before injection	5,800,000	80		Negative
	" 9	After injection	5,600,000	75		
	" 10	" "	4,500,000	68		
	" 11	" "	4,000,000	60	*B. muris* bodies (occasional)	
	" 12	" "	4,200,000	62		
	" 13	" "	3,300,000	60		
	" 14	" "	3,000,000	50	Occasional *B. muris*	Positive culture
	" 16	" "	4,000,000	60		
	" 21	" "	7,000,000	100		

bin dropped to 50 per cent and the red cell count to 3,000,000 per c.mm. *Bartonella muris* bodies were occasionally seen. On the 5th day the blood culture was positive for *Bartonella muris* (see Table II).

Two adult rabbits injected repeatedly with large amounts of the culture intravenously showed no evidence of disease.

Infection of 3 Week Old Guinea Pigs with Bartonella muris Cultures

Three 3 week old guinea pigs and three adult guinea pigs were injected intraperitoneally with 0.5 cc. of a young culture of *Bartonella muris* on leptospira medium. The three young animals became severely anemic within 24 hours. The adult animals remained unaffected. The anemia continued for 3 to 5 days with recovery of the animals. The *Bartonella* bodies were found on the red cells,

very sparsely scattered.[2] The organism was recovered from the blood on the 2nd and 5th days of the anemia. In one instance (see Table III) the hemoglobin

TABLE III

3 week old guinea pig injected with 1 cc. culture *Bartonella muris*. (Sample protocol.)

Date		Red cell count	Hb (Dare)	*Bartonella* bodies	Blood culture
1932			*per cent*		
Mar. 29	Before injection	4,900,000	80		
" 29	Injected with 1 cc. culture				
" 30	After injection	2,500,000	48	Occasional *B. muris* body	
" 31	" "	2,200,000	25	" "	Positive culture *B. muris*
				Killed. Autopsy: fatty liver, watery blood, marked anemia, congested spleen	

TABLE IV

7 week old mouse injected with 0.3 cc. culture of *Bartonella muris*. (Sample protocol.)

Date		Red cell count	Hb (Dare)	*Bartonella* bodies	Blood culture
1932			*per cent*		
May 27	Before injection	5,500,000	120		
" 28	After injection	5,000,000	110		
" 29	" "	5,200,000	110		
" 30	" "	6,000,000	105		
" 31	" "	6,500,000	105	Occasional	
June 1	" "	3,100,000	40	"	
" 2	" "	2,700,000 (Killed)	29	"	Positive

dropped to 25 per cent and the count to 2,200,000 per c.mm. Autopsy of this animal (killed by heart puncture) revealed the pathological picture of *Bartonella*

[2] Rybinsky (6) observed bodies on the red blood cells of adult guinea pigs that were previously repeatedly injected with trypan blue, and of guinea pigs infected with *T. brucei*. He describes these as round and oval, staining a dark violet color with Giemsa and measuring 1 to 2 microns in size. He suggests the name of *Bartonella ukrainica* for this type of *Bartonella* infection.

TABLE V

The Production of Bartonella muris Anemia in Adult Splenectomized Rats of Non-Carrier Stock (Wistar) Following Injections of Bartonella muris Cultures. Cultures Were Injected 2 Days after Splenectomy

Injected with 5 cc. culture	Length of time after splenectomy	Blood counts													
		Days after injection													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
	days														
1	2	100% 7500T	95% 7500T	90% 6000T	90% 6700T	105% 7500T	100% 7000T	110% 7000T	110% 7800T	105% 7500T	100% 7200T	78% 6200T	78% 7500T	90% 6700T	90% 7100T
2	2	105% 7600T	90% 7300T	80%* 6300T	76%* 5900T	62%* 4500T	80% 4750T	100% 5800T	50% 4800T	50% 4000T	95% 4500T	105% 5800T	110% 7000T	105% 6500T	
3	2	99% 8000T	110% 8200T	100% 6900T	90% 7100T	90% 8000T	85% 7900T	105% 7800T	110% 7000T	108% 7200T	110% 6900T	85% 5800T	100% 6800T		
4	2	105% 8700T	110% 8500T	85% 6400T	90% 6900T	105% 8500T	110% 9000T	105% 8500T	102% 8200T	80% 6200T	90% 7500T	105% 8500T	110%		
5	2	110% 8500T	88% 7500T	70%* 6500T	80% 6500T	80% 6200T	85% 6000T	105% 7500T	105% 8200T	115% 8000T	105% 7900T				
6	2	110% 8600T	88% 8500T	70% 7000T	75% 5500T	75% 6000T	70% 6000T	95% 8200T	110% 8000T	110% 8100T	105% 7900T				
7	2	110% 8600T	88% 8500T	78% 4900T	69% 5000T	65%* 5000T	68% 6000T	62% 5500T	100% 6200T	110% 8500T	105% 8200T	100% 7800T			

8 (control)	2	110% 8250T	105% 7800T	105% 8200T	100% 7900T	105% 8100T	110% 8600T	105% 8000T	100% 7900T	102% 8150T	105% 7800T	105% 8100T	105% 8250T	
9	2	95% 8700T	105% 10000T	110% 9000T	105% 8000T	110% 80000	108% 8500T	105% 8300T	105% 8200T	100% 7500T	102% 7800T	110% 8300T	105% 8000T	
10	2	95% 8500T	100% 9000T	95% 9000T	100% 8700T	105% 8200T	100% 8000T	110% 8200T	100% 7800T	110% 7500T	105% 8200T	105% 9000T	105% 9200T	

The letter T is used in place of the last three zeros of the red cell count.
The hemoglobin is expressed in percentages as calculated from readings with the Dare hemoglobinometer.
* *Bartonella muris* bodies occasionally seen on red cells.

muris anemia with marked fatty changes in the liver, congested spleen and anemia of the organs. The blood had a watery consistency.

This is the first instance to our knowledge of the production of *Bartonella muris* anemia in the guinea pig. The strain recovered from the guinea pig was infectious for young rats.

The organism is non-pathogenic for adult guinea pigs.

Infection of Young White Mice with Bartonella muris Cultures

The susceptibility of white mice for *Bartonella muris* infection has been reported by Adler (7) and by Lwoff and Vaucel (3).

Four white mice were injected intraperitoneally with 0.3 cc. of a *Bartonella muris* culture. In three instances occasional *Bartonella* bodies were seen on the red cells after a period of 3 to 6 days. They occurred primarily in the red cells. In one instance a severe anemia occurred on the 5th day following the injection of the culture. The count dropped to 2,700,000 red cells per c.mm. and the hemoglobin to 29 per cent. The culture of the blood was positive for *Bartonella muris* on the 6th day.

Infection of Adult Wistar Splenectomized Rats of Non-Carrier Stock with Bartonella muris Culture

The Wistar stock are non-carriers of *Bartonella muris* infection. The adult Wistar rat is markedly resistant to infection, though it may be infected by the injection of large amounts of blood of anemic splenectomized rats (8). The splenectomized adult Wistar rat is very susceptible to infection with *Bartonella muris* anemia (9). The rats used in the test recorded were of Wistar stock, transported from the Wistar Institute to the laboratory of the Montefiore Hospital Country Sanatorium, 50 miles from the city, and bred there, out of all possible contact with infected stock. They were sent to our laboratory at the time of use and isolated.

With the early cultures a definite anemia was not produced in splenectomized adult Wistar rats though the organism was recovered from the blood stream several days following the injection of the culture. After 10 subcultures and 1 animal passage, however, the organism produced a mild anemia in five of seven adult rats of the Wistar stock after an incubation period of 3 to 5 days.

The hemoglobin dropped to 60 per cent and the red cells to 4,200,000 from 8,500,000 per c.mm. (see Table V). The organism was recovered from the blood. The white cell count rose from 10,500 to 65,000. This marked leukocytosis is characteristic of spontaneous *Bartonella muris* anemia. Occasional *Bartonella muris* bodies were found in red cells. They were not found in all cases and were never numerous. When large amounts of a culture were injected into Wistar unsplenectomized adult rats, they died within 24 hours of a severe toxemia. The organs showed some congestion. Adult unsplenectomized rats of carrier stock remain unaffected by injections of the organism.

Serological Tests

Agglutination tests with homologous sera of rabbits repeatedly injected with 1 cc. of a heavy suspension of washings from blood agar slants proved negative.

Two rabbits were injected intravenously at 5 day intervals with 1 cc. of a heavy suspension of washings of cultures on blood agar. 5 days after the third injection the serum was tested against a suspension of living *Bartonella muris*. The suspension was obtained by repeatedly washing the bacteria obtained from blood agar slants. After four washings with distilled water the bacteria were resuspended in distilled water and utilized in agglutination tests. Negative results were obtained even in high concentration of the serum. Negative results were obtained with serum of infected rats and with serum of rats spontaneously infected with *Bartonella muris*.

Complement fixation tests were carried out using the bacterial suspension as antigen.

One-quarter of the anticomplementary amount of the antigen was used in the test. The serum of carrier rats, of spontaneously infected splenectomized rats, of rats infected with *Bartonella muris* cultures, of Wistar non-carrier rats and of homologous rabbit serum were tested. Positive fixation of complement was obtained in homologous rabbit serum and in anemic rats in very low dilutions (1/40). Similar results were obtained with antigen prepared by prolonged aqueous extraction of bacterial suspensions in a Soxhlet apparatus.

DISCUSSION

Bartonella muris and *Bartonella bacilliformis* resemble each other much both morphologically and culturally. Noguchi isolated *Bartonella bacilliformis* from the blood of a patient suffering with Oroya fever and reproduced the disease in monkeys (1). He established the identity of verruga peruana and Oroya fever by producing Oroya

infection in a monkey with macerated material of a verruga nodule and reisolated *Bartonella bacilliformis* from the blood of the infected monkey (10, 11). In our experience *Bartonella muris* is somewhat less delicate in appearance than *Bartonella bacilliformis*[3] and stains more deeply. The growth on leptospira medium is definitely more luxuriant and the growth in subcultures appears in 48 hours, whereas *Bartonella bacilliformis* appears in 8 to 10 days. The growth on blood hormone agar during the first few days is similar to that of *Bartonella bacilliformis* but the *muris* colonies soon coalesce and form a tenacious film.

The similarity of *Bartonella muris* anemia in the rat and Oroya fever of human beings and the morphological and cultural characteristics of the two organisms suggest a close relationship between *Bartonella muris* and *Bartonella bacilliformis*. Further work will be undertaken to determine the possible rôle of the rat in the epidemiology of Oroya fever and verruga peruana.

SUMMARY

1. *Bartonella muris* has been isolated in pure culture on Noguchi's leptospira medium from the blood of the splenectomized adult rat suffering with *Bartonella muris anemia.*

2. *Bartonella muris* is a small, actively motile, Gram-negative bacillus. It grows best on media containing blood and on Noguchi's leptospira medium. The optimal temperature for growth is 20–25°C. It produces neither gas nor acid on media containing sugars. It does not hemolyze blood in artificial media. Viability of the cultures in leptospira media was maintained for 36 days.

3. With this culture a severe anemia was produced in rats weighing 30 gm., with the occasional appearance of *Bartonella muris* bodies on the red cells. The anemia occurred within 24 hours, and lasted for 3 to 5 days with recovery of the rat. *Bartonella muris* was recovered in pure culture from the blood of these animals. The blood of these rats was infectious for other 30 gm. rats.

4. 3 week old rabbits were infected with cultures of *Bartonella muris*, a severe anemia resulting after an incubation period of 48 hours. The

[3] We are indebted to Dr. Peter Olitsky of The Rockefeller Institute for Medical Research for a culture of *Bartonella bacilliformis.*

organism was recovered from the blood on the 5th day after injection. *Bartonella muris* is non-pathogenic for adult rabbits.

5. A severe anemia was produced in young guinea pigs with cultures of *Bartonella muris* within 48 hours. The organism was recovered on the 2nd and 5th days after injection. Postmortem examination revealed changes in the organs similar to those found in splenectomized rats suffering with spontaneous *Bartonella muris* anemia.

6. The infection was reproduced in white mice. In one instance a severe anemia developed on the 5th and 6th days. The organism was recovered on the 6th day.

7. The anemia was produced in splenectomized adult rats of non-carrier stock. The organism was recovered from the blood stream of these rats. A marked leukocytosis was noted (65,000) at the peak of the anemia as is found in the spontaneous disease in infected splenectomized adult rats.

8. Serological tests have thus far failed to demonstrate the production of agglutinins, though complement-fixing antibodies are present in homologous sera in low dilutions.

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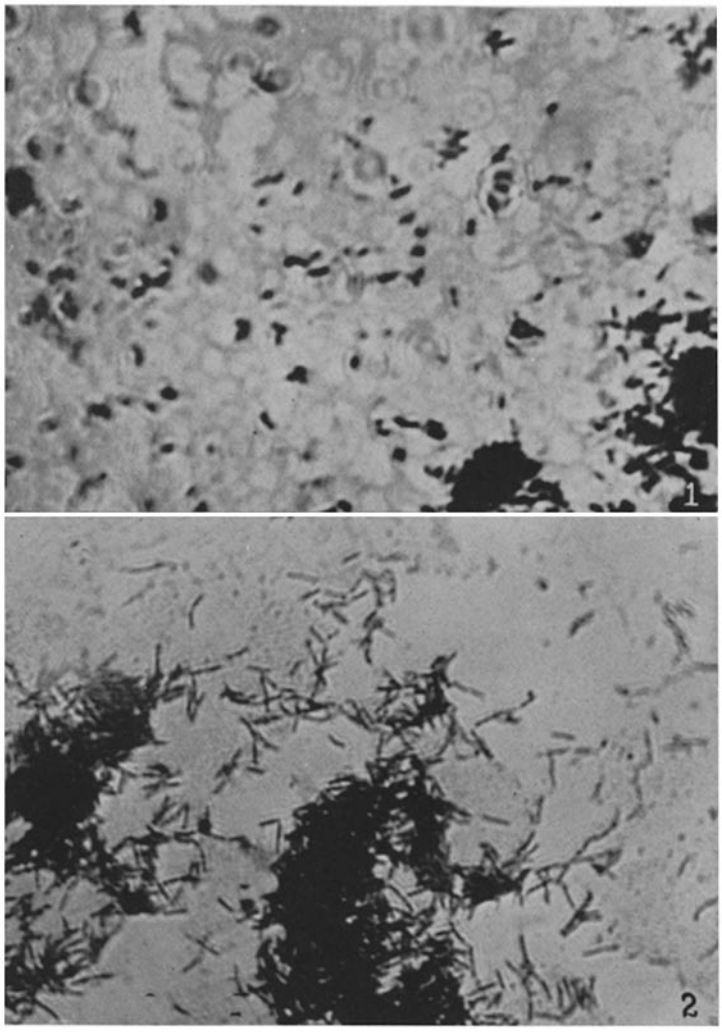
EXPLANATION OF PLATE 36

FIG. 1. *Bartonella muris* growth in leptospira medium. Gram stain. × 1200.
FIG. 2. *Bartonella muris* growth on blood hormone agar. Gram stain. × 1200.



(Marmorston-Gottesman and Perla: Etiology of *Bartonella muris* anemia)