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ETIOLOGY OF OROYA FEVER.

I. Cultivation of Bartonella bacilliformis.

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Plates 33 to 35.

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The microbic incitant of Peruvian Oroya fever has been recognized by previous investigations.

Barton,[1] especially, detected the bacilliform "X" bodies which occur constantly in the red corpuscles of cases of the infection and distinguished them accurately from the non-specific basophilic rods and granules present in the red corpuscles in other diseases. Strong, Tyzzer, Sellards, Brues, and Gastiaburu,[2] who also detected the bacilliform bodies, regarded them as motile microorganisms probably of protozoan nature which invade both red corpuscles and vascular endothelium; and they proposed for them the name of *Bartonella bacilliformis*. *Bartonella bacilliformis* would seem to be the specific cause of Oroya fever, as malaria plasmodia are for malaria, despite the fact that the organism has been found in certain cases of verruga peruviana. While this association of two clinically dissimilar diseases has proved disturbing, Peruvian investigators had connected Oroya fever with verruga long before the bacilliform organism was discovered. According to Odriozola,[3] verruga may be present when Oroya suddenly supervenes, or both maladies may arise simultaneously, or verruga may follow Oroya. These facts have led many Peruvian investigators to regard verruga and Oroya as different manifestations of the same disease, a conclusion supported by the heroic deed of Carrion, who, believing in a common etiology for verruga and Oroya, submitted himself to inoculation with verruga, the result being the development of a clinical case of fever, accompanied by the usual severe anemia, to which he succumbed.

We are in a relatively favorable situation today for deciding on the relationship of Oroya fever and verruga. The improvements in bac-

[1] Barton, A. L., *Crón. méd.*, 1909, xxvi, 7.

[2] Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaburu, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

[3] Odriozola, E., La maladie de Carrion, Paris, 1896.

teriologic technique of the last few years have led to the cultivation of a number of microorganisms previously resisting culture. It was regarded as desirable that these methods be applied to Oroya fever. There can no longer be doubt that it and verruga can exist together, either one preceding in appearance the other. Moreover, it has been thoroughly demonstrated[4,5] that the monkey may be successfully inoculated intradermally from cases of verruga. The possibility exists, therefore, of testing by inoculation the power of cultures derived from cases of Oroya fever to set up the lesions of verruga in animals.

We have applied the method originally devised for the cultivation of leptospiras[6] and leishmanias[7] to blood derived from a case of Oroya fever (Santa Anna 15) in the service of Dr. Olaechea, of the Dos de Mayo Hospital, Lima. Through the generous permission of Dr. Olaechea, a blood sample was withdrawn on September 4, 1925; the patient died 2 days later from the severe progressive anemia usual in the disease.

Several culture tubes were inoculated with the fresh citrated blood at Lima, and these tubes, together with the remainder of the citrated blood, were brought back to New York, the date of departure from Lima being September 9, and of arrival in New York September 21. The blood was kept at refrigerator temperature during the entire period after its collection from the patient.

Bacterial contaminations occurred in the original culture tubes during the journey, but renewed attempts at cultivation with the citrated blood yielded a pure culture of *Bartonella bacilliformis*, which has been employed in the studies to be reported here. The media listed in Table I were used, with the results noted.

Each tube was inoculated with 0.05 cc. of the citrated blood, placed at 37°C. for 4 days, and then removed to room temperature for 4 days or more. Only a trace

[4] Jadassohn, G., and Seiffert G., *Z. Hyg. u. Infectionskrankh.*, 1910, lxvi, 247.

[5] Mayer, M., Rocha Lima, H., and Werner, H., *Münch. med. Woch.*, 1913, lx, 739.

[6] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13; *J. Trop. Med.*, 1925, xxviii, 185.

[7] Noguchi, H., *Proc. Internat. Conf. Health Prob. Trop. America*, Kingston, Jamaica, British West Indies, July 22 to August 1, 1924, published in Boston, 1924, p. 455.

of opalescence occurred on the surface of the tubes of leptospira medium, and they were at first set aside as negative. On reexamination a few days later, however, numerous minute organisms were recognized. Examination by dark-field microscope, as well as staining with Giemsa's and Gram's solutions, were utilized for the detection of the organism.

TABLE I.

1. Leptospira medium. 0.9 per cent NaCl..... 800 parts. Fresh rabbit serum..... 100 " 2 per cent nutrient agar (pH 7.4)..... 100 " Rabbit hemoglobin (made by laking 1 part of defibrinated blood with 3 parts of distilled water)..... 10–20 "	Growth on surface layer.
2. Same, containing fresh tissue.	" " " "
3. Leptospira medium containing glucose, maltose, inulin.	" " " "
4. Same, plus fresh tissue.	" " " "
5. Horse blood agar slant. Defibrinated horse blood added to melted 2 per cent nutrient agar (pH 7.4) to give concentration of 20 per cent.	" " " "
6. Same, containing glucose, maltose, inulin.	" " " "
7. Plain agar slant.	No growth.
8. Plain broth.	" "

Bartonella bacilliformis in Culture.

Growth and Morphology.—Bartonella bacilliformis grows on solid or semisolid media containing blood of the rabbit, horse, or man; in a fluid medium it never multiplies sufficiently to be readily recognized. No growth can be obtained on ordinary slants or broth. Growth on blood slants is difficult to recognize, since the colonies are extremely minute and transparent, hardly visible to the naked eye (Fig. 2). Cultures on the semisolid leptospira medium, when the number of organisms introduced is large, are more readily detected by

a grayish haziness or punctiform colonies in the top layer. After 1 to 2 weeks at 28°C. the growth extends as deep as about 1 cm. below the surface (Fig. 1, *a*), and resembles somewhat in appearance the growth of leptospiras. When, on the other hand, the number of organisms inoculated is small, several very minute, discrete, grayish white granules first appear, and as time goes on each colony becomes gradually surrounded by a hazy zone (Fig. 1, *b*). This phenomenon is never seen in cultures of any of the leptospiras. The organisms grow at 37°C. as well as at 25–28°C., but at the lower temperatures they continue to grow and survive for a much longer time. In this respect *Bartonella bacilliformis* behaves much as do various flagellates and leptospiras, which can be most conveniently cultivated and maintained at the lower temperatures. No growth takes place under anaerobic cultural conditions.

The hydrogen ion concentration in which growth occurs lies between pH 6.8 and 8.4, but the organisms grow best at pH 7.8. No morphological variations due to varying the hydrogen ion concentration have thus far been detected. None of the common carbohydrates (dextrose, saccharose, galactose, maltose, levulose, xylose, lactose, mannose, mannitol, dulcitol, arabinose, raffinose, rhamnose, dextrin, inulin, salicin, and amygdalin) are fermented. Erythrocytes contained in culture medium are not hemolyzed by the growth of the organism.

When grown on blood slants at 25°C. the organisms are motile for many days and are rather uniformly rod- or spindle-shaped. Aggregates of a few to several hundred organisms are usually seen, both in fresh preparations by dark-field illumination, and in stained ones. By dark-field illumination, most of the young forms are seen to be actively motile, those aggregated into masses moving along slowly, the free individuals dashing swiftly across the field. In the leptospira medium are found small coccobacillary forms fairly well separated from one another when embedded in the medium. Many aggregates of organisms are also observed (Fig. 3). They are less motile in this medium and lose their motility usually within 1 to 2 weeks at 28°C. Pleomorphism prevails, and huge abnormal spirochete-like flagella are visible under the dark-field microscope in such cultures (Fig. 4, *a*). The normal flagella of active organisms are invisible except when stained by special methods (Fig. 4, *b*).

The organisms appear larger in fresh preparations viewed by dark-field illumination than after staining, and young forms show a definite double contour (Figs. 3, *a* and *b*). The rods are not always straight; sometimes they show a slight bend, and they are often massed together in aggregates of from two or three to a hundred. In older cultures the double contour effect is lost, the organisms appearing as decidedly thinner rods, agglomerated into masses in innumerable numbers (Fig. 3, *c*). They are now no longer motile.

The microorganism is Gram-negative and stains reddish violet with Giemsa's solution (Figs. 5 and 6). After Giemsa's staining, however, the sharp outlines are lost, and the organisms appear ill defined as if through degeneration. Minute punctiform, spindle-shaped, or oval elements, some in chains or in irregular entanglements, may be found. There are also rather large and irregular forms, densely packed in masses and taking a deeper stain. All the forms seen within the red corpuscles of Oroya patients (Fig. 7, *a*, *b*, and *c*) are also to be found in cultures. The stained specimens of cultures vary in length from 0.3 to 2.5μ—exceptionally to 3μ—and in width from less than 0.2 to 0.5μ. Chains of several individuals are also seen. Coarse oval forms measure about 0.7 by 1.2μ.

Inoculation Effects.

Inoculation of the cultures into various animals (ringtail, *rhesus*, Java, and green monkeys, dogs, rabbits, mice, rats, and guinea pigs) were undertaken, but the results thus far have been definite only in the *rhesus* monkeys. The following protocols and charts represent the findings in these animals.

Macacus rhesus 1 (Chart 1; Fig. 9). November 16, 1925, received intravenously 2 cc. of cultures from leptospira medium (9 days old) and sugar blood slant (4 days old). At the same time the eyebrows were intradermally inoculated with the same material at two sites on each side. The animal remained apparently well until 8 days later, when it seemed less active. The temperature never rose above 104.8°F. at any time during the 55 days of observaton, the type of fever being irregularly remittent and very mild. Areas of induration became evident at the sites of intradermal injection after 10 to 14 days, and some of these developed into reddish nodules approximately 6 by 4 mm. in diameter.

Blood was withdrawn 13, 22, 33, and 43 days, after the inoculation. Cultures made with the blood yielded, on each occasion, a pure growth of *Bartonella bacilli-*

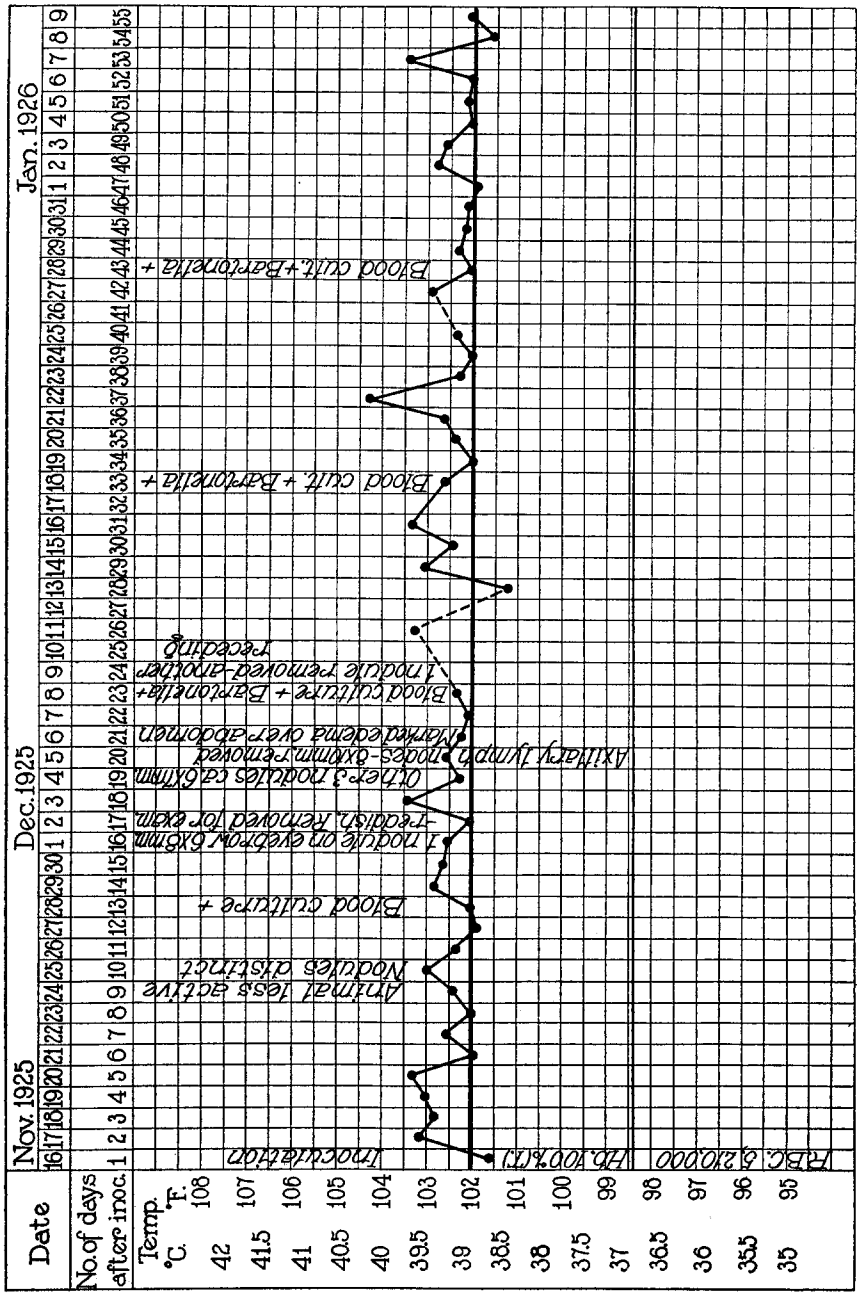


CHART 1. *Macacus rhesus* 1.

formis. A small number of intracorpuscular forms of the organism were demonstrated in blood smears (Fig. 8, *a*). One of the nodules, which was excised[8] 16 days after inoculation, showed typical infiltration of mononuclear phagocytes but was negative for *Bartonella bacilliformis.* The blood withdrawn 23 days after inoculation was transferred to three normal *Macacus rhesus* monkeys.

Macacus rhesus 2 (Chart 2). December 8, 1925, received intravenously 6 cc. of a mixture containing cultures of the 1st, 2nd, and 3rd generations derived from the blood of *Macacus rhesus* 1, as well as citrated blood withdrawn from the same animal on two occasions (the day of inoculation and 11 days previously). At the same time the left eyebrow was injected intradermally with the mixture and the right eyebrow with the emulsion of the nodule freshly excised from *Macacus rhesus* 1. The temperature rose to 104.3°F. on the 5th day and remained at about that point for 3 days. The fever in this animal fluctuated irregularly, the highest point reached being 105°F. on the 23rd day. There were several times during the 50 days of observation when the temperature fell to subnormal. The culture made with blood withdrawn on the 1st day of fever yielded a pure growth of *Bartonella bacilliformis*, as did also those made with blood withdrawn 9, 11, 21, 28, and 36 days after inoculation; and typical forms of the organism were found in a few of the red corpuscles (Fig. 8, *b*). At the sites of inoculation on the left eyebrow definite nodules had developed after about 28 days and these continued to increase in size during the following weeks.

Macacus rhesus 3 (Chart 3; Fig. 16). Inoculated at the same time and in the same way as *Macacus rhesus* 2. The course of disease was similar to that in the foregoing animal, except that the febrile reaction was much more violent during the first 5 weeks. It was similarly irregular, however, and remittent. The two sites of intradermic inoculation with culture on the left eyebrow began to show induration within about 10 to 14 days. Only one of the two inoculations on the right eyebrow (inoculated with the emulsion of a nodule of *Macacus rhesus* 1) gave rise to a nodule, and induration became evident only after 28 days, the evolution of the lesion being much slower than that of nodules produced with culture material. All three nodules were still increasing in size when examined 45 days after inoculation. One became pedunculated and cherry-red after 2 months and subsequently softened at the center. A yellowish fluid began to exude from it 68 days after the inoculation.

Macacus rhesus 4 (Chart 4; Fig. 17). The date of injection and the material injected were the same as in the foregoing two animals. The reaction was somewhat more severe than in the others, the temperature being 104°F. on the day following the injection and reaching 105.4° on the 7th day. On the 16th day the fever was interrupted, the temperature falling to slightly subnormal for 1 day. 13 days of fever followed, and the temperature was 103.4° on the 23rd day, when the animal died while under anesthesia during removal of one of the nodules. No sign of tuberculosis could be found at autopsy. The spleen was enlarged. The

[8] All operations were performed under ether aneshesia.

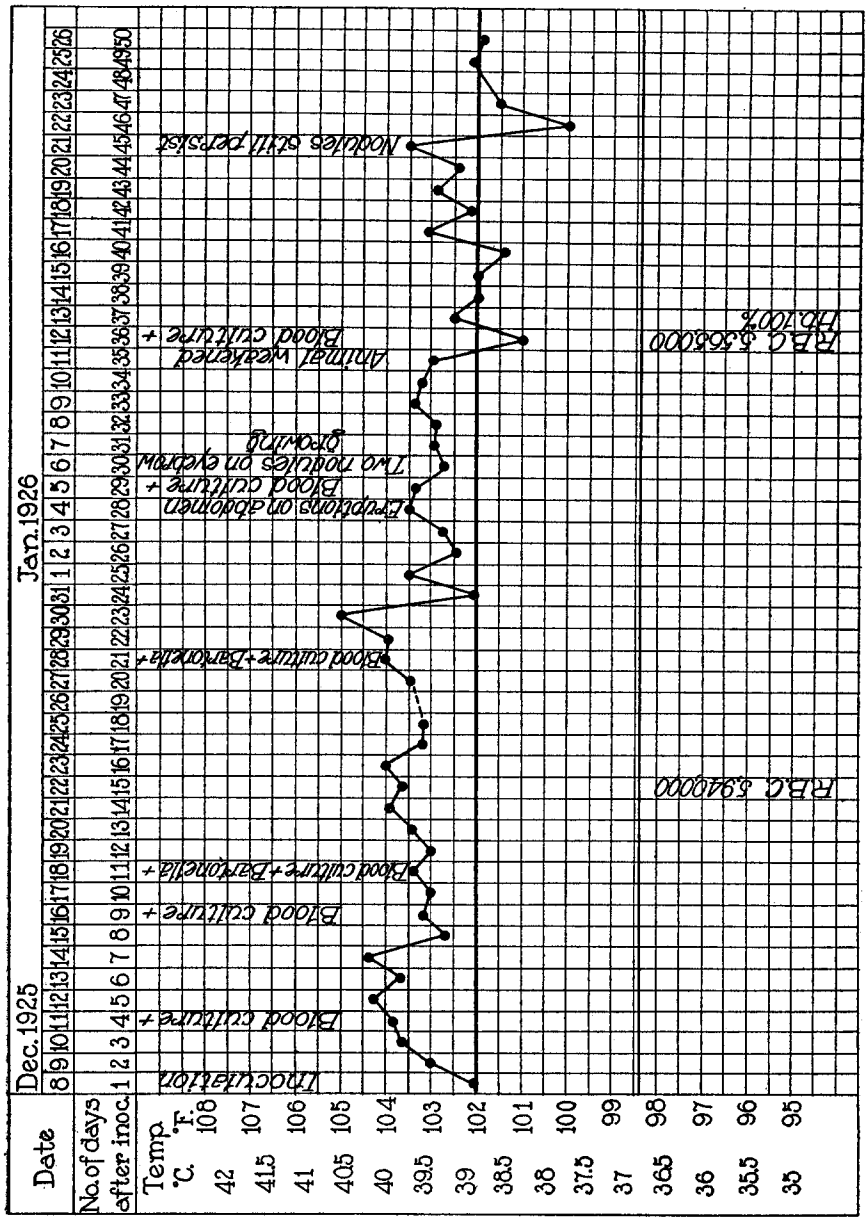


CHART 2. *Macacus rhesus* 2.

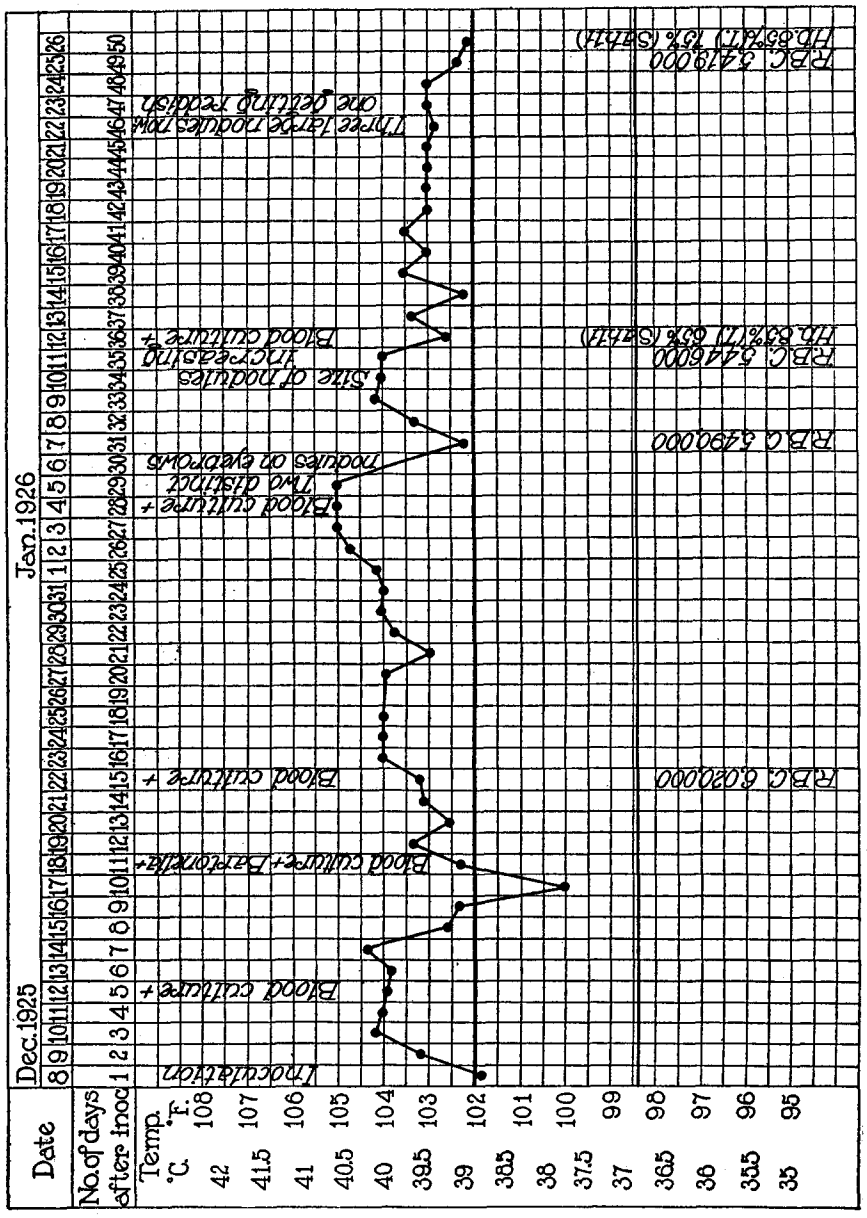


CHART 3. *Macacus rhesus* 3.

lymph glands were generally enlarged, but there were no lesions in the lungs. Pure cultures of *Bartonella bacilliformis* were obtained from blood withdrawn 5, 7, 11, 14, and 21 days after the inoculation, and from blood, spleen, and lymph nodes taken at autopsy. *Bartonella bacilliformis* was also found in small number in the red corpuscles (Fig. 8, *c*). The nodules excised 13 and 23 days after inoculation showed edema, and endothelial and capillary proliferation (Figs. 9 and

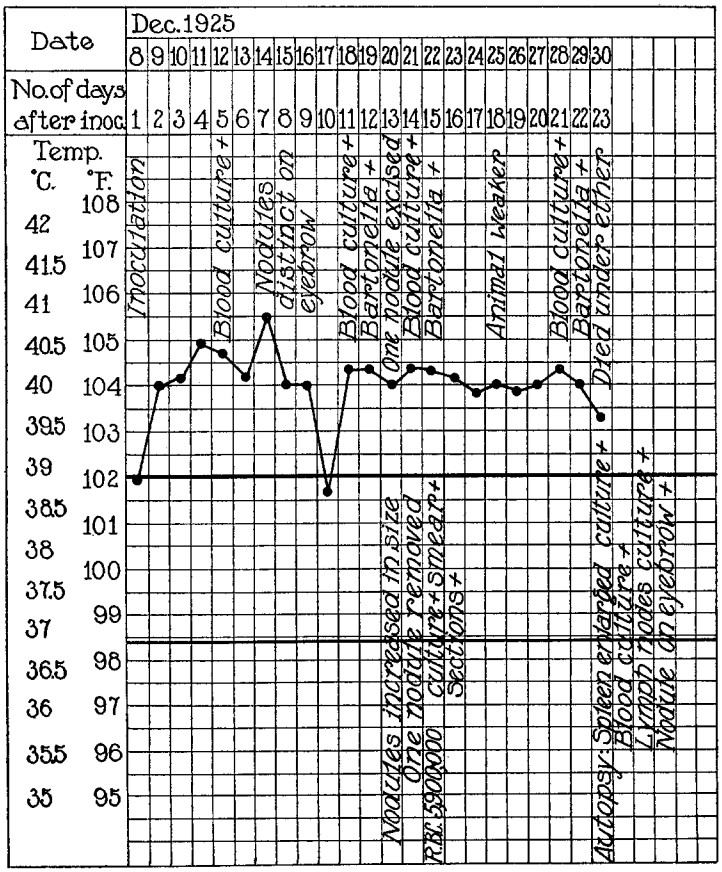


CHART 4. *Macacus rhesus* 4.

10), the microorganisms being demonstrated in cultures, smears, and sections (Figs. 13 to 15), as well as by dark-field examination of the fresh emulsion (Fig. 11). No tubercle bacilli were found in any of the tissues examined.

Further observations on the surviving monkeys, as well as the results

of other animal inoculations, will be reported later. The findings here described suffice to show that a culture of *Bartonella bacilliformis* obtained from the blood of an Oroya fever patient induces in young *rhesus* monkeys, when inoculated intravenously, a peculiar, irregularly remittent, and sometimes severe type of fever, and when inoculated intradermally into the eyebrow, gives rise to a nodule rich in cellular elements and capillary formation. Control inoculations have been made with the culture medium employed for growing the organism, and with cultures killed by heating at 65°C. for 15 minutes. Neither of these materials led to any of the changes induced by the living organisms.

Bartonella bacilliformis persists in the blood of the infected animals for many weeks, as shown by blood cultures and smears. Intracellular forms of the organism are readily found in the proliferated endothelial cells in the nodules, from which pure cultures are also to be obtained.

The infection in monkeys, unlike Oroya fever in man, has not thus far given rise to anemia, and the number of infected red corpuscles is very small, prolonged search being required to detect one. The lymph glands and spleen, however, become enlarged, and pure cultures of *Bartonella bacilliformis* have been isolated from them.

Inoculation into *rhesus* monkeys of the citrated human blood from which our cultures were derived, after it had been kept 18 days in the ice box, induced no apparent symptoms, but since the blood of these animals was not tested culturally the success or failure of the inoculations cannot be definitely stated. It is possible that overwhelming numbers of young and active organisms, such as are present in cultures, are necessary to break down the normal resistance of the monkeys, which is considerable. The relatively high resistance of such animals, as compared with man, may explain also the fact that pronounced anemia does not occur in the experimental infection. Further study upon these points is necessary.

Filtration experiments with cultures have been negative.

SUMMARY.

A pure culture of a microorganism resembling in morphology and pathogenic action *Bartonella bacilliformis* has been obtained from blood

taken during life from a case of Oroya fever which ended fatally. The blood taken at Lima into citrate solution and transported to New York at refrigerator temperature yielded positive cultures 28 days after its withdrawal from the patient.

The strain of *Bartonella bacilliformis* thus isolated grows well on the semisolid leptospira medium, and also on slant agar containing animal blood. The initial growth is not readily recognizable to the naked eye, but the presence of the organisms can be determined by means of the dark-field microscope and by Giemsa and Gram staining methods. No growth has been obtained on the more ordinary culture media. The organism is an obligate aerobe, is Gram-negative, and under certain cultural conditions motile. All the forms which have been described as occurring in human red corpuscles may be found in the cultures, and in addition many granular and coarsely irregular forms have been met with.

The inoculation of cultures of *Bartonella bacilliformis* into *Macacus rhesus* produces infection and gives rise to effects which differ with the mode of inoculation. The intravenous injection of the culture into young macaques induces a prolonged irregularly remittent fever. The organism can be cultivated from the blood over a long period, and it has been detected within the red corpuscles of the monkeys, reproducing the precise appearances observed in human cases of Oroya fever.

The intradermal injection of the culture into the eyebrow of young macaques gives rise to nodular formations rich in new blood vessels and showing the bacilliform organism within the endothelial cells. From the experimentally induced nodules cultures of the organism are readily recovered.

EXPLANATION OF PLATES.

PLATE 33.

FIG. 1. Appearance of growth of *Bartonella bacilliformis* on leptospira medium inoculated (*a*) with 0.01 cc. of the citrated blood of *Macacus rhesus* 4 and (*b*) with 0.00001 cc. Fig. 1, *b* shows several discrete hazy colonies. Cultivation at 28°C. for 14 days.

FIG. 2. Appearance of surface colonies of *Bartonella bacilliformis* on sugar blood agar plate, cultivated at 28°C. for 4 days. The colonies were so minute and so

nearly transparent that they had to be photographed by passing the light obliquely over the plate. Hence they appear as white spots, owing to the reflection of light by them. × 4½.

FIG. 3. Dark-field views of *Bartonella bacilliformis* in cultures grown at 28°C. (*a*) on agar blood slant for 5 days, (*b*) on leptospira medium for 7 days, and (*c*) on agar blood slant for 5 days. Organisms with definite double contour are shown in (*a*) and (*b*), while (*c*) illustrates the tendency of the organisms to agglomerate into huge masses, in which it is difficult to distinguish individuals. × 1000.

FIG. 4, *a*. Two detached gigantic involuted flagella of *Bartonella bacilliformis* in a culture grown on leptospira medium for 10 days at 28°C. × 1000.

FIG. 4, *b*. Young organisms with flagella (6 days on blood slant at 28°C.). Flagella stain. × 1000.

FIG. 5. Culture forms of *Bartonella bacilliformis* grown on blood agar slant at 28°C. for 4 days. Giemsa's stain. × 1000.

FIG. 6. Cultures forms of *Bartonella bacilliformis* grown on leptospira medium at 28°C. for 5 days. Giemsa's stain. × 1000.

PLATE 34.

FIG. 7, *a* to *c*. *Bartonella bacilliformis* in human red blood corpuscles. The smear was made from the blood of the patient from whom our strain was isolated. Several extracorpuscular forms are also shown. Giemsa's stain. × 1000.

FIG. 8. *Bartonella bacilliformis* in red blood corpuscles of (*a*) *Macacus rhesus* 1, (*b*) *Macacus rhesus* 2, and (*c*) *Macacus rhesus* 4, all of which were inoculated with culture. Giemsa's stain. × 1000.

FIG. 9. *Macacus rhesus* 1, showing three well developed nodules on the left eyebrow 16 days after intradermic inoculation of culture of *Bartonella bacilliformis*.

FIG. 10. Section showing the structure of one of the nodules produced by intradermic injection of culture of *Bartonella bacilliformis* isolated from the blood of *Macacus rhesus* 1. The nodule was removed 23 days after the inoculation. Regaud's fixation, Giemsa's stain. × 182.

FIG. 11. *Bartonella bacilliformis* in an emulsion of one of the nodules of *Macacus rhesus* 4. Dark-field view. × 1000.

FIG. 12. *Bartonella bacilliformis* in a smear of the same emulsion. Giemsa's stain. × 1000.

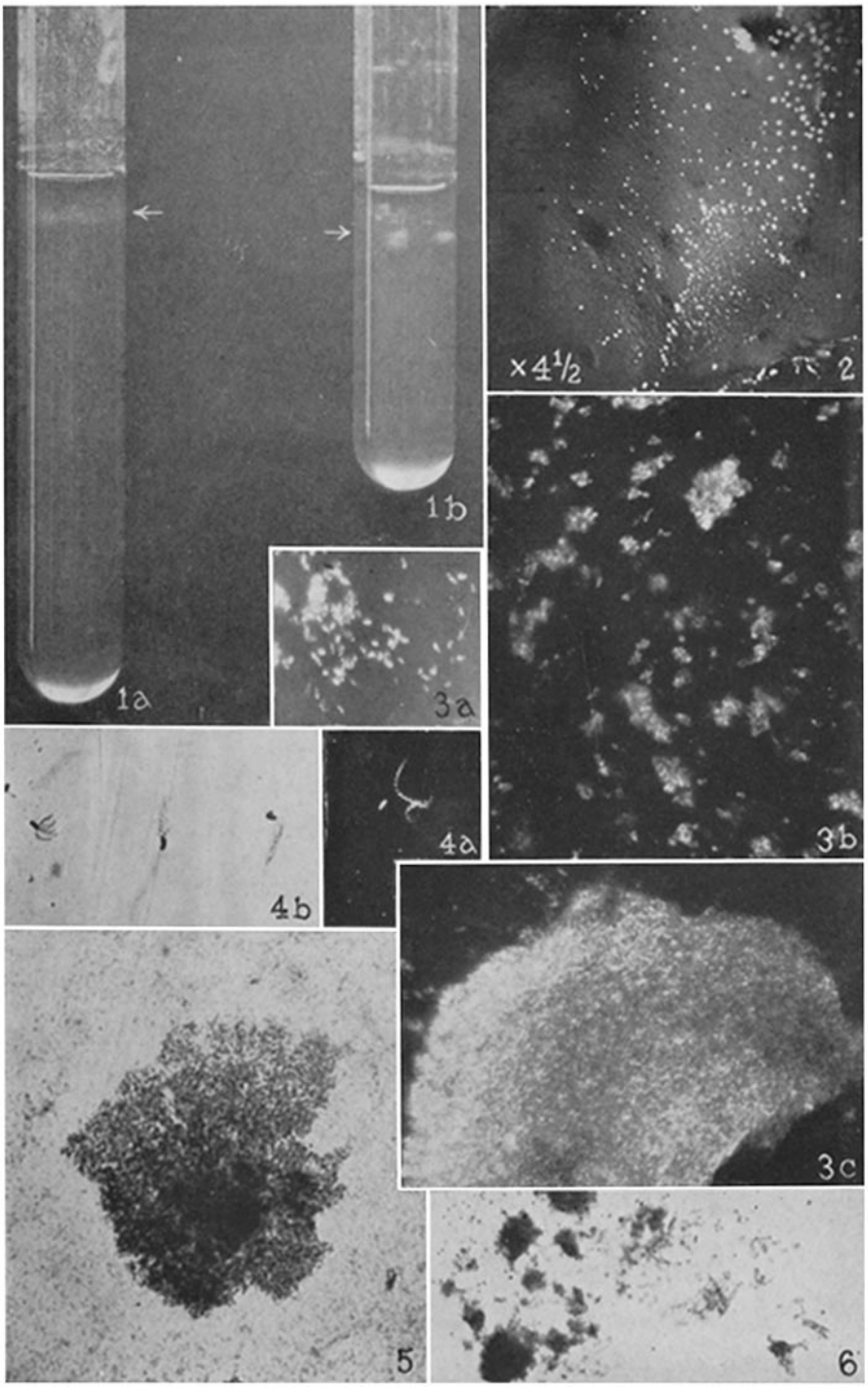
FIGS. 13 to 15. *Bartonella bacilliformis* in the angioblasts and endothelial cells of newly forming capillaries. Sections fixed in Regaud's and stained with Geimsa's solution. × 1000.

PLATE 35.

FIG. 16. *Macacus rhesus* 3. The nodule on the right eyebrow developed after the intradermic inoculation of an emulsion of a nodule excised after 16 days from *Macacus rhesus* 1. The picture shows the natural size and color of the nodule 45

days after inoculation, when it was excised for histological and cultural study and for further passage. The two nodules on the left eyebrow resulted from the injection of cultures obtained from the blood of *Macacus rhesus* 1. The picture shows their size and color 2 months after inoculation. The pedunculated nodule shows a tiny opening at the apex; it subsequently became softened at the center, and 68 days after inoculation a yellowish fluid began to exude. It was excised the following day (February 15, 1926) for sectioning, cultural studies, and transfer. Life size.

FIG. 17. *Macacus rhesus* 4. The appearance of the nodules 23 days after intradermic inoculation of the same culture as was injected into *Macacus rhesus* 3. The nodule near the median line stands out prominently and shows two round indurations. The epidermis is expanded and shiny. There was no softening. The nodule at the outer end of the left eyebrow was less circumscribed, but it was more deep seated, extending into the surrounding subcutaneous tissues. It was of firm consistency, like the others. The sites of inoculation, on the right eyebrow, of the emulsion of nodule tissue from *Macacus rhesus* 1 show scarcely a trace of induration. Life size.



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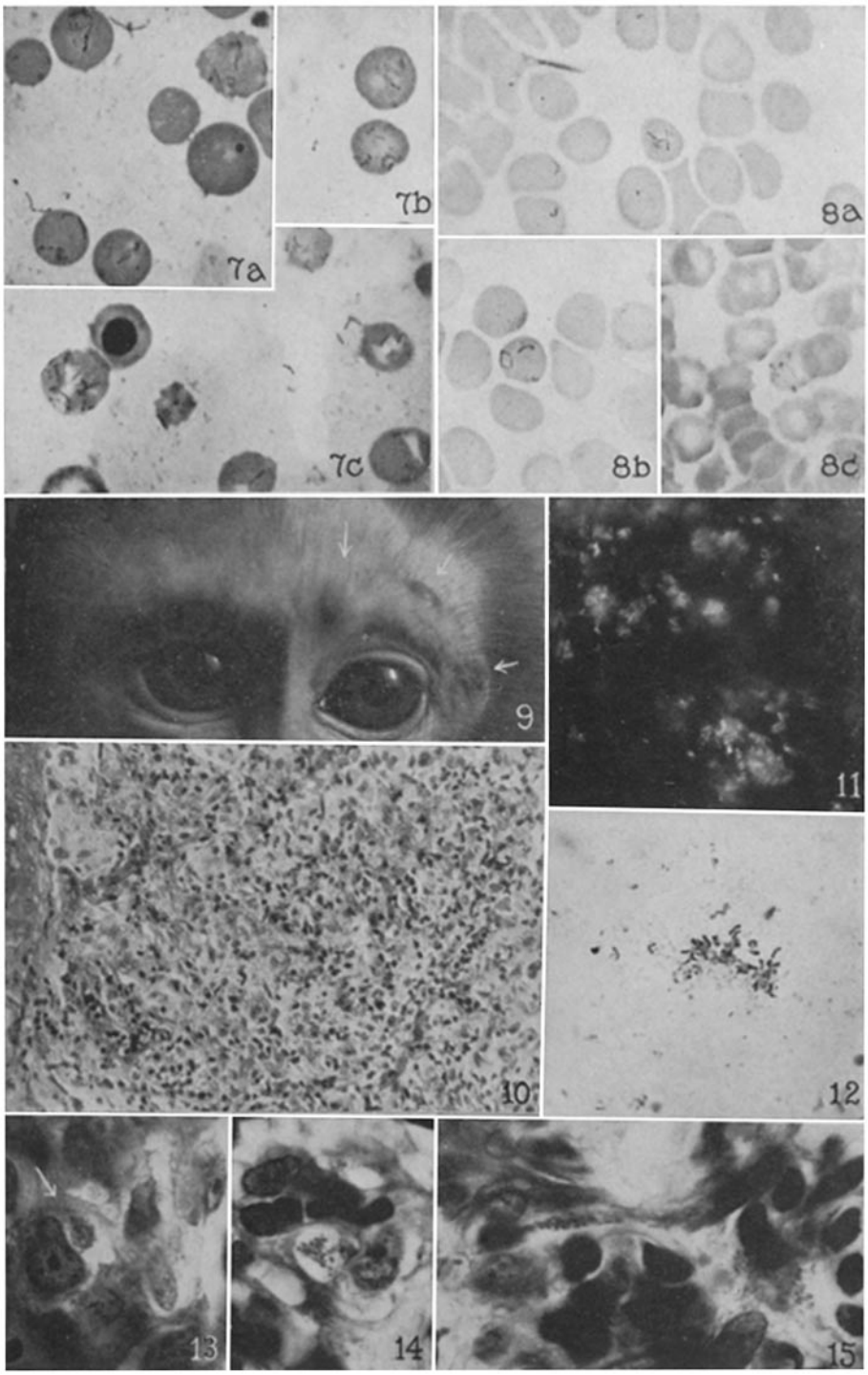

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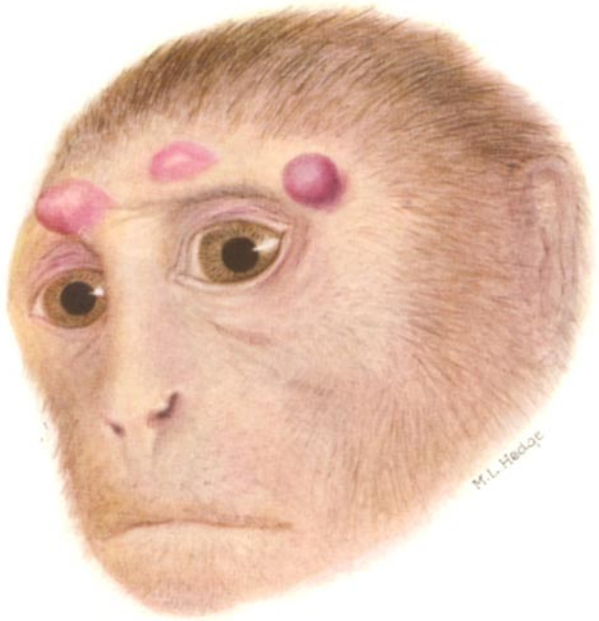

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(Noguchi and Battistini: Etiology of Oroya fever. I.)