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ETIOLOGY OF OROYA FEVER.

XIV. THE INSECT VECTORS OF CARRION'S DISEASE.

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PLATES 45 TO 47.

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It is desirable to present the two parts of this investigation in a single paper, since they bear on each other so closely that to publish them separately will call for considerable repetition of statement. The origin of the studies which have led to the results here presented is to be found in the earlier papers of this series (1), and in the work of Townsend (2), who concerned himself especially with the insect vector of the disease embraced under the names of Oroya fever and verruga peruana. Since the observations given in this paper were completed after the death of Dr. Noguchi, who planned the experiments, we wish to state briefly the circumstances surrounding the investigation.

The earlier papers of the series established *Bartonella bacilliformis* as the bacterial incitant of Oroya fever (Carrion's disease) and verruga peruana. The etiology and much of the pathology of these manifestations of a single infectious disease were made clear by the experimental studies (Noguchi) carried out between 1925 and 1928. The essential fact which remained to be determined was the precise mode of infection in the two maladies for which the history and clinical observation had indicated an insect vector. Townsend's studies of the distribution of the disease and the nocturnal nature of its origin had led him to the decision that the vector belonged to the class of phlebotomi. Indeed, he had gone so far as to designate the vector as *Phlebotomus verrucarum*.

The cooperation of the International Health Division of the Rockefeller Foundation was secured in the field investigation of insects in the verruga zones in Peru. Various insect species were collected by one of us (Shannon), identified as far as possible on the grounds, the identifications being completed afterwards in the United States, and sent by ship to New York, where they were tested for infectivity on monkeys according to Dr. Noguchi's plans (Tilden and Tyler). The procedure employed in this testing was as follows:

The insects were collected without the use of chemicals and sealed alive in sterile tubes, which were either dry, or contained a piece of absorbent cotton moistened with sterile citrate solution (about half of each shipment was sent dry, the other half in moist condition). Collections were made near the time of sailing of the fast boats to New York, and shipments were placed in the steamer's refrigerator during transit.

The method of determining the presence of *Bartonella bacilliformis* in the insects was to inject a saline suspension of the crushed bodies intradermally, sometimes also intravenously, into monkeys (*Macacus rhesus*) and to make cultures of the blood at intervals of from one to four weeks later, irrespective of the occurrence of local lesions or fever. A given lot of insects was crushed in 0.9 per cent sodium chloride and injected intradermally into one or two sites on the shaved abdominal skin of two monkeys at least. Because of the differences in susceptibility of individual monkeys, duplicate tests were necessary.

The culture technique was the same as that used in earlier work on Carrion's disease (3). The blood was withdrawn from the monkey into an equal part of 2 per cent citrate in 0.9 per cent sodium chloride, and ascending dilutions in saline (1:10, 1:100, 1:1,000, 1:10,000, and 1:100,000) were inoculated into the semisolid leptospira medium (4) in amounts of 0.2 cc. One tube of the medium was inoculated with a drop of the undiluted citrated blood. The cultures were kept at 30°C. By the end of a two to three week period the positive cultures can usually be picked out by their macroscopic appearance, but even microscopic examination may fail to reveal a positive culture, and subculture is often desirable (5). The growth may be so slight in the initial culture as to be easily mistaken for the haze which develops in a tube of sterile medium after standing at 30°C.

Although *Bartonella bacilliformis* was detected only in the phlebotomi, it is desirable to state that other insects collected (Shannon) in the verruga zone were inoculated into *rhesus* monkeys in the manner of the phlebotomi and the blood cultures carried out in the same way as for the latter. In no instance was *Bartonella bacilliformis* isolated in these cultures. A list of the insects with which no results were obtained follows:

Ticks.—*Ornithodoros megnini* (on burros), *Argas* sp.? (on burros, birds, bats), Tick larvae (genus and species?) on lizard.

Mites.—*Tarsotomus* sp. (on ground), *Trombidium* n.sp. (on ground), *Geckobia* sp. (on lizard), *Geckobiella* sp. (on lizard and geckos).

Lice.—*Trichodectes ovis* (on sheep).

Fleas.—*Pulex irritans* (on man), *Ctenocephalus canis* (on cat, dog, and man), *Rhopalopsyllus* (on dog and guinea pig).

Bedbugs.—*Cimex lectularius*.

Mosquitoes.—*Anopheles pseudopunctipennis*, *Culex quinquefasciatus* (*fatigans*).

Buffalo gnats.—*Simulium escomeli*, *Simulium* sp. (on burros).

Midges.—*Forcipomyia utae*, *Forcipomyia townsendia*.

Muscidæ.—*Stomoxys calcitrans*.

Hippoboscidae.—*Melophagus ovinus* (on sheep).

Streblidae.—3 genera, one species each (on vampire bats).

Note should be made of the fact that Townsend (2) regarded the lizard as the natural reservoir of the incitant of verruga peruana, for the reason that he detected intracorpuseular bodies in the blood cells, which he identified with Barton's rods. Hence the red mites (*Trombidium*, *Tarsotomus*, *Geckobia*, *Geckobiella*), some of which were obtained from geckos (*Phyllodactylus reisi*), as well as the blood of two geckos, were injected into monkeys. Cultures prepared from the blood of these monkeys remained sterile.

Phlebotomi of Verrugas Cañon.

One of us (Shannon) spent from March to July, 1928, in the Rimac verruga zone, Peru. As many varieties of insects as possible were collected (6) from this zone and sent to The Rockefeller Institute in New York to be tested upon monkeys. It is desirable to state that Townsend was the first to implicate phlebotomus with the transmission of verruga peruana. His studies, conducted between 1912 and 1914, led him to decide, on ecological and experimental grounds, that a species of gnat, later called *Phlebotomus verrucarum* Townsend, was the vector of the disease.

Our studies revealed three species of *Phlebotomus* in the verruga zone. Two of the species had a wide and the third a limited distribution only in the zone. It is significant that of the three, only the two which occurred in considerable numbers were found on inoculation to yield *Bartonella bacilliformis*.

In this paper brief descriptions only will be given of the three species, based upon the characters of the males. All three species belong to the subgenus *Brumptomyia* (França and Parrot), which may be described as follows:

Basal segment of the upper appendage of the male terminalia with a well defined sub-basal tuft of hairs on the inner surface; the distal appendage either with four well developed spines, the fifth one weak, or with five strong spines; median appendage simple, without spines, lower appendage unarmed. The abdominal hairs are suberect to erect; length of the upper branch of upper forked vein longer than the petiole preceding the fork.

Ph. noguchii and *Ph. peruensis* are described here for the first time (Shannon).

Key to the Males.

1. Distal segment of upper appendage with four well developed spines, the fifth (an apical one) being very slender and hair-like; petiole of upper forked

cell slightly longer than that section of the first longitudinal vein which overlaps the upper branch of the second vein. . . . *Ph. verrucarum* Townsend.

2. Distal segment with five well developed spines, both apical ones being equally strong.

(a) Distal segment with two submedian spines, a third located slightly distal of the middle of the segment, the fourth and fifth forming an apical pair; petiole of the upper forked cell much longer than that section of the first vein which overlaps the upper branch of the second vein. (Type locality, Verrugas Cañon, Department of Lima, Peru)

Ph. noguchii Shannon.

(b) Distal segment with two submedian spines, a subapical one and an apical pair; that section of the first vein overlapping the upper branch of the second is distinctly longer than the length of the petiole of the upper forked cell. (Type locality, Matucana, Department of Lima, Peru)

Ph. peruensis Shannon.

The females of *Phlebotomus peruensis* can be separated from those of *verrucarum* and *noguchii* by differences in the arrangement of the wing veins, but definite characters have not yet been found whereby the females of *verrucarum* and *noguchii* may be positively identified. Approximate identification of the females which were sent to New York for bacteriological study was made on the basis of (1) average differences in size, *verrucarum* being in general smaller than *noguchii*, and (2) habitat. All males found in houses proved to be *verrucarum*, hence all females found in-doors were assumed to belong to this species. All the *noguchii* males were found out-of-doors, in excavations and natural cavities, where they were three times as numerous as *verrucarum* males.

Before leaving this description, it may be well to bring together certain accepted facts with reference to the epidemiology of Carrion's disease and certain known habits of the phlebotomi. It is admitted that the disease is contracted only in certain limited areas in Peru, and that infection, with possibly rare exceptions, takes place only at night. This infection may be acquired indoors or in localities remote from human habitations and at any time of the year.

With these facts in mind, it would seem to follow that the insect vector must be (a) common blood sucker of man; (b) restricted to the verruga zone; (c) nocturnal in habit; (d) capable of breeding in varied localities, so that adults, which have restricted flight, may be everywhere present, and (e) active throughout the year.

All these conditions are fulfilled by phlebotomi and not, as far as determined, by other insects of the verruga zones.

Finally, it may be recorded that we (Shannon and assistant) safely

spent from 2 to 5 nights a week for 4 months in the verruga zone after taking adequate precaution to protect ourselves from bites of phlebotomi.

Experiments with Phlebotomi.

Eighteen special lots of phlebotomi, prepared and shipped as described, were inoculated into monkeys (Tilden and Tyler). The material in each instance was introduced intradermally at several sites on the shaved skin of the abdomen, and was also applied to a scarified area of the abdominal skin. Occasionally an intravenous injection was also made.

Lots 1, 2, 9, and 14 were pooled, ground in a mortar with 0.9 per cent saline solution, and injected, Apr. 25, 1928, into two monkeys (*Macacus rhesus* I-3 and I-4).

Lot 1, collected Mar. 9, 1928, out-of-doors in Matucana. 3 females and 4 males of *Ph. noguchii*, *Ph. verrucarum*, and *Ph. peruensis*.

Lot 2, collected Mar. 9, 1928, out-of-doors in Matucana. 2 females, 18 males, *Phlebotomus*, chiefly *noguchii*.

Lot 9, *Ph. verrucarum* collected Mar. 20, 1928, in house in Verrugas Cañon. 40 females.

Lot 14, *Ph. verrucarum*, collected Mar. 26, 1928, in Verrugas Cañon. 24 females.

Lot 20. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected Apr. 9, 1928, consisted of about 20 females. A saline suspension of the crushed insects was injected May 31, 1928, into two monkeys (*M. rhesus* I-7 and I-8). I-8 also received 1 cc. of the suspension intravenously.

Lots 27 and 28. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected May 1 and May 8, 1928. 10 to 12 females. A saline suspension of the crushed insects was injected June 13, 1928, into two monkeys, *M. rhesus* I-17 and I-5.

Lots 29, 30, and 38. *Ph. verrucarum*, collected May 1 and May 8, 1928, 15 to 20 females. Saline suspension injected June 13, 1928, into *M. rhesus* I-16 and *M. rhesus* I-6.

Lots 40, 41, and 44. *Ph. verrucarum*, collected June 9, 11, and 18, 1928, both in houses and out-of-doors, in Verrugas Cañon. These lots comprised about 100 females. Saline suspension injected July 14, 1928, into *M. rhesus* I-26 and I-27. All the specimens which came in moist condition were covered with green mold.

Lots 39 and 45. *Ph. noguchii*, collected June 6 and June 19, 1928, out-of-doors in Verrugas Cañon. The number of insects was small (about 25 females), and the specimens which came in moist condition were covered with green mold. Saline suspension inoculated July 14, 1928, into *M. rhesus* I-28 and I-29.

Lots 42 and 46. *Ph. peruensis*, collected June 12 and 19, 1928, out-of-doors in Matucana. The specimens which came in moist condition were covered with green mold. 8 females. Saline suspension inoculated July 14, 1928, into *M. rhesus* I-30 and I-31.

Lot 43. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, collected June 12, 1928, out-of-doors in Matucana. Saline suspension inoculated Aug. 13, 1928, into two monkeys, I-33 and I-34.

Lot 51. *Ph. verrucarum*, collected during the last week in July, 1928. 6 females. Saline suspension inoculated Aug. 13, 1928, into *M. rhesus* I-37.

Lot 54. *Ph. noguchii*, with possibly a few *verrucarum* intermixed, 8 to 10 females, collected during the last week in July out-of-doors in Verrugas Cañon. Saline suspension inoculated Aug. 14, 1928, into *M. rhesus* I-38 and I-39.

The first material inoculated, which contained all three species of *Phlebotomus* (Lots 1, 2, 9, and 14) yielded positive results.

Strain 1, from Lots 1, 2, 9, and 14.

M. rhesus I-3 and I-4, inoculated intradermally Apr. 25, 1928. No local lesions developed at the sites of inoculation. *M. rhesus* I-3 had a temperature of 104°F. on May 14, 19 days after inoculation, and blood was withdrawn on that day. *Bartonella bacilliformis* was obtained in culture from 1:10, 1:100, and 1:1,000 dilutions of the blood. The temperature reached 104°F. again several times, but blood culture was negative on May 29 and June 30. Blood was taken from *M. rhesus* I-4 at the same time as from I-3, but cultures remained negative.

Inoculation of Cultures from M. rhesus I-3.—*M. rhesus* I-14 and *M. rhesus* I-15 (Fig. 1) were inoculated June 5, 1928, with the 20 day culture of *Bartonella bacilliformis* obtained from the blood of *M. rhesus* I-3 and a 5-day subculture. The culture, which was, as usual, diluted with an equal part of saline for inoculation, was also applied to a scarified area on the abdominal skin. Tiny nodules were observed in *M. rhesus* 15 at the sites of intradermal inoculation on June 11 (16 days after inoculation), and 5 days later they were well developed, and one was excised¹ for examination and transfer. *Bartonella bacilliformis* was obtained from a 1:1,000 dilution of blood withdrawn June 16, and from a 1:100,000 dilution of the nodule suspension. By June 22 the scarified area showed small nodules. The photograph was taken 3 days later (Fig. 1). By June 28 the lesions had disappeared. *M. rhesus* I-14 had almost continuous high fever (104° to 106°F.) from June 8 to 28 and again from July 16 to 23, but no local lesions developed, and blood culture was negative 13, 31, and 55 days after inoculation.

The strain of *Bartonella bacilliformis* obtained from these first lots of phlebotomi was carried through two animal passages by direct transfer and has since been maintained by alternate generations in culture and

¹ All operations were carried out under ether anesthesia.

monkey. The usual course of verruga of moderate severity (7) has been observed in the animals (Fig. 8), with the exception of one monkey of the first passage (*M. rhesus* I-1), which had an unusually severe cutaneous reaction (Figs. 6, 7), not unlike that which had been induced in one of the monkeys (*M. rhesus* 18) of an early experiment (Noguchi (8)). This animal was acutely ill over a period of two weeks but recovered.

Histological study of the nodular tissue from I-15 and I-1, made by Dr. Henry R. Muller, shows the characteristic proliferation of endothelial cells and the formation of new capillaries. *Bartonella bacilliformis* was detected in some instances within the endothelial cells.

The second lot of phlebotomi tested, which probably consisted chiefly of *Ph. noguchii* but may have contained a few *verrucarum*, also yielded a strain of *Bartonella bacilliformis*.

Strain 2 from Lot 20.

M. rhesus I-7 and I-8 were inoculated May 31, 1928, intradermally, and I-8 received 1 cc. of the suspension in the left saphenous vein. The animals were bled on June 11 and again on June 30. The blood of I-8 yielded cultures of *Bartonella bacilliformis* in 1:100,000 dilution on June 11 and in 1:100 dilution on June 30; no cultures were obtained from the blood of I-7. Neither animal developed lesions at the sites of inoculation, but I-8 had almost continuous fever (104° to 105.2°F.) from June 4 to June 28.

Inoculation of Culture from M. rhesus I-8.—*M. rhesus* I-22 and I-23 were inoculated on June 22, 1928, intradermally, with a 10-day-old culture from the blood of *M. rhesus* I-8. The culture was also applied to a scarified area on the abdomen. The nodules showed at the sites of intradermal inoculation after about 10 days, and blood withdrawn after 2 weeks yielded cultures of *Bartonella bacilliformis* in 1:10,000 dilution. The nodule excised¹ on July 6 for examination and transfer yielded cultures in a 1:100 dilution. The lesions were considerably larger and more extensive in I-23 (Fig. 3), and the edema of the abdominal wall developed early and became marked. Regression of the lesions began about four weeks after inoculation and within four weeks recovery was practically complete. The course of disease was almost afebrile in both animals.

Further inoculations, first with nodular tissue from I-22 and I-23, and later with cultures from the blood of passage animals, showed that this strain of *Bartonella bacilliformis*, like Strain 1, was moderately virulent, inducing pronounced local lesions and moderate anemia.

One animal of the series died in 33 days, after an afebrile course of disease, during which moderately severe anemia had been observed.

Lots 27 and 28 (chiefly *Ph. noguchii*) and Lots 29, 30, and 38 (*Ph. verrucarum*) yielded negative results, as did also Lots 40, 41, 44, and 51 (*Ph. verrucarum*), Lots 42 and 46 (*Ph. peruensis*), and Lot 43 (*Ph. noguchii*).

From Lots 39 and 45, which probably consisted of *Ph. noguchii* alone, a third strain of *Bartonella bacilliformis* was obtained.

Strain 3, from Lots 39 and 45.

M. rhesus I-28 and I-29 were inoculated intradermally on July 14, 1928. Monkey I-28 showed no fever at any time, and blood cultures were negative 1, 2, and 3 weeks after inoculation. Monkey I-29 had fever (104.2° to 105.2°F.) 3 days after inoculation, which continued for a week with one day of remission. The blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100 on four occasions, 7, 13, 23, and 31 days after inoculation, but there was no reaction at the sites of injection.

Inoculation of Cultures from I-29.—*M. rhesus* I-44 was inoculated intradermally and by scarification on Aug. 16, 1928, with a culture 14 days old from the blood of *M. rhesus* I-29. 1 cc. of the culture was also injected into the saphenous vein. Nodules developed in 2 weeks (Figs. 5 and 9), and the scarified area presented the characteristic miliary eruption. The blood was positive in a dilution of 1:10,000 at this time. From Sept. 7 to 19 there was marked fever (104° to 105.2°F.).

M. rhesus I-45 was inoculated at the same time and in the same manner as I-44. The intradermal nodules attained a diameter of only 0.5 cm., and the blood was positive in a dilution of 1:100. Fever existed (104.2° to 105.4°F.) from Sept. 10 to 13, was followed by two days of subnormal temperature, and death of animal on Sept. 16. Autopsy (Dr. Muller) revealed nothing abnormal except in the spleen, which contained numerous pale areas 2 to 3 mm. in diameter. Film preparations were negative for tubercle bacilli, and microscopic examination disclosed infarcts such as are found in the spleen in human (9) cases of Oroya fever, and in cases of the experimental disease (10).

Later passage of Strain 3 produced local lesions of very large size (2 to 3 cm. in diameter), but no unusual systemic effects.

Lot 54, which consisted chiefly, perhaps wholly, of *Ph. noguchii*, also yielded *Bartonella bacilliformis*.

Strain 4, from Lot 54.

M. rhesus I-38 was injected intradermally on Aug. 14, 1928, and intravenously (1 cc. of the saline suspension into the left saphenous vein). From Aug. 22 to

Aug. 29 the temperature was 104°F., but blood culture made Aug. 28 was negative. It was also negative on Sept. 10, but blood taken on Sept. 25, when the temperature was 104.2°F. yielded *Bartonella bacilliformis* in 1:10 and 1:100 dilutions after 13 days incubation. The intradermal mixtures produced no lesions. *M. rhesus* I-39, inoculated at the same time as I-38, and with the same material, showed a rise of temperature (104.2° to 104.8°F.) on three occasions, but blood cultures made on Aug. 28, Sept. 10, and Sept. 25 were negative.

Inoculation of Cultures from I-38.—*M. rhesus* I-58 was inoculated on Oct. 10, 1928, with 15-day culture from the blood of *M. rhesus* I-38. Small nodules appeared at the sites of intradermal injection after 7 days and were well advanced after 16 days (Fig. 5). The abdominal wall became oedematous and the area of scarification showed miliary nodules in addition to which three or four small eruptions arose outside the inoculated areas. Blood culture was positive in dilutions up to 1:10,000, 12 days after inoculation. The animal died on the 18th day, when the local lesions were still actively progressing. Histological examination of tissues by Dr. Muller revealed the characteristic zonal necrosis around the central vein in the liver, with extensive invasion by polymorphonuclear leucocytes. The spleen showed no lesions. The various skin nodules were histologically characteristic of verruga in the monkey.

Further inoculations with cultures of Strain 4 yielded similar results. In one animal (*M. rhesus* S-7) the local lesions reached large size.

The results of the inoculations are summarized in Tables I to V.

Exposure of Monkeys to Bites of Phlebotomi.

Six *rhesus* monkeys were exposed (Shannon) for several weeks to natural infection, three in an excavation in Verrugas Cañon, where *Ph. noguchii* was fairly common, and three in a house where *Ph. verrucarum* was abundant. These animals were brought to The Rockefeller Institute on Aug. 13. Blood withdrawn on three occasions failed to yield cultures of *Bartonella bacilliformis*, and only one of the animals failed to respond to subsequent inoculation of virulent cultures or passage virus. The result therefore was regarded as negative.

Immunity.

Seven of the monkeys which had developed verrucous lesions and blood infection with *Bartonella bacilliformis* following inoculation with the Phlebotomus strains and in which the lesions had regressed, were subsequently tested for immunity by reinoculation. Similar immunity tests were made on three monkeys which had received crushed

TABLE I.
Inoculations of Crushed Phlebotomi.

<i>M. rhesus</i> No.	Date 1928	Lot No.	Method of inoculation	Local lesions	Blood culture
I-3	Apr. 25	1, 2, 9, 14 <i>verrucarum</i> <i>noguchii</i> <i>peruensis</i>	Multiple intradermal Scarification	—	+
I-4	Same	Same	Same	—	—
I-7	May 31	20 <i>noguchii</i> (few <i>ver-</i> <i>rucarum?</i>)	Multiple intradermal Scarification	—	—
I-8	Same	Same	Same, also intravenous	—	+
I-16	June 13	29, 30, 38 <i>verrucarum</i>	Multiple intradermal Scarification	—	—
I-6	Same	Same	Same	—	—
I-17	Same	27, 28 <i>noguchii</i> (few <i>ver-</i> <i>rucarum?</i>)	Same	—	—
I-5	Same	Same	Same	—	—
I-26	July 14	40, 41, 44 <i>verrucarum</i>	Same	—	—
I-27	Same	Same	Same	—	—
I-28	Same	39, 45 <i>noguchii</i>	Same	—	—
I-29	Same	Same	Same	—	+
I-30	Same	42, 46 <i>peruensis</i>	Same	—	—
I-31	Same	Same	Same	—	—
I-33	Aug. 13	43 <i>noguchii</i> (few <i>ver-</i> <i>rucarum?</i>)	Same	—	—
I-34	Same	Same	Same	—	—
I-37	Same	51 <i>verrucarum</i>	Same	—	—
I-38	Same	54 <i>noguchii</i> (few <i>ver-</i> <i>rucarum?</i>)	Same, also intravenous	—	+
I-39	Same	Same	Same	—	—

TABLE II.

Strain 1, from Lots 1, 2, 9, 14 (Ph. verrucarum, Ph. noguchii, Ph. peruensis).

<i>M. rhesus</i> No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-14	June 5	Culture from I-3	Intradermal Scarification	—	—
I-15	Same	Same	Same	+++	+
First passage					
I-18	June 16	Nodule susp. I-15	Same	++++	+
I-19	Same	Same	Same	++++	+
I-1	Same	Same	Same	++++	+
Second passage					
I-12	July 6	Nodule susp. I-1	Same	++++	+
I-13	Same	Same	Same	+++	+
Third passage (via culture)					
I-40	Aug. 16	14-day culture I-12	Same, also intra- venous	++++	+
I-41	Same	Same	Same	++++	+

TABLE III.

Strain 2 from Lot 20 (Ph. noguchii—few verrucarum?).

<i>M. rhesus</i> No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-22	June 22	10-day culture from I-8	Intradermal Scarification	++++	+
I-23	Same	Same	Same	++++	+
First passage					
I-24	July 6	Nodule susp. I-23	Same	++++	+
I-25	Same	Same	Same	+++	+
Second passage (via culture)					
I-42	Aug. 16	14-day culture from I-24	Same, also intrave- nous	++++	+
I-43	Same	Same	Same	++++	+

TABLE IV.
Strain 3 from Lots 39 and 45 (*Ph. noguchii*).

<i>M. rhesus</i> No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-44	Aug. 16	14-day culture from I-29	Intradermal Scarification	++++	+
I-45	Same	Same	Intravenous Same	++++	+
First passage					
I-55	Sept. 13	Nodule susp. I-44	Intradermal Scarification	+++	+
I-56	Same	Same	Same	++	-
I-34	Oct. 22	20-day culture from I-45	Same	+++	+
Second passage (via culture)					
S-6	Dec. 15	25-day culture from I-34	Same	++++	+

TABLE V.
Strain 4 from Lot 54 (*Ph. noguchii*—*few verrucarum?*).

<i>M. rhesus</i> No.	Date 1928	Material inoculated	Mode of inoculation	Local lesions	Blood culture
I-58	Oct. 10	15-day culture from I-38	Intradermal Scarification	+++	+
First passage (via culture)					
I-17	Nov. 5	14-day culture from I-58	Same	++++	+
Second passage (via culture)					
S-7	Dec. 15	18-day culture from I-17	Same	++++	+

TABLE VI.
Immunity Tests.

<i>M. rhesus</i> No.	First inoculation				Immunity test			
	Date, 1928	Material inoculated	Local lesions	Blood culture	Date, 1928	Material inoculated	Local lesions	Blood culture
I-18	June 16	Nodule susp. I-15 (Str. 1)	++++	+	Sept. 13	Nodule susp. I-41 (Str. 1)	-	-
I-13	July 6	Same	++++	+	Same	Same	-	-
I-3	Apr. 25	Phlebotomi Lots 1, 2, 9, 14	-	+	Same	Same	++++	-
I-53 Control					Same	Same	++++	+
I-8	May 31	Phlebotomi Lot 20	-	+	Sept. 13	Nodule susp. I-43 (Str. 2)	++++	+
I-25	July 6	Nodule susp. I-23 (Str. 2)	++++	+	Same	Same	-	-
I-23	June 22	Blood culture I-8 (Str. 2)	++++	+	Same	Same	-	-
I-11	July 6	Nodule susp. P. 5*	++++	+	Same	Same	-	-
286 I-54 Control	June 1	Culture P. 5*	++++	+	Same Same	Same Same	- +++	- +
I-1	June 16	Nodule susp. I-15 (Str. 1)	++++	+	Sept. 26	Culture P. 5*	-	-
I-19	June 16	Same	++++	+	Same	Same	-	-
I-29	July 14	Phlebotomi Lots 39, 45	-	+	Same	Same	+++	+
I-57 Control					Same	Same	+++	+

* Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

phlebotomi without developing skin lesions which reacted as do previously untreated animals. Table VI summarizes the results.

Morphology.

No morphological or cultural differences could be detected between the Phlebotomus strains and the human strains of *Bartonella bacilliformis*. Cultures seven days old of the four strains, grown on horse blood agar slants, were stained to bring out the unipolar flagella (one to four) which are characteristic of *Bartonella bacilliformis* (Figs. 11, 13, 15, 17), the films being made on the same slide, in order that the stained preparations might be comparable. Cultures of the same age but grown on leptospira medium were used for similar comparative preparations which were stained by Gram's method, with fuchsin as the counterstain (Figs. 10, 12, 14, 16).

SUMMARY AND CONCLUSIONS.

With a view to determining the mode of infection in Carrion's disease, a study of the blood-sucking insects found in the districts of Peru where the disease prevails has been carried out, through the cooperation of The Rockefeller Institute and the Rockefeller Foundation. The material studied included ticks, mites, midges, lice, fleas, bedbugs, mosquitoes, buffalo gnats, horse-flies, "sheep ticks," 3 species of Streblidae, and 3 species of Phlebotomus, including *Phlebotomus verrucarum* Townsend and two new species which have been named *Phlebotomus noguchii* and *Phlebotomus peruensis*. The insects were collected without the use of chemicals, were prepared for transportation in such a manner as to prevent drying, and were shipped under conditions of refrigeration to New York, where they were inoculated into monkeys. The plan followed was to inject saline suspensions of the crushed insects intradermally into *rhesus* monkeys and to make cultures of the blood of the animals at intervals of 1 to 6 weeks after inoculation.

The only class of insects in which the presence of *Bartonella bacilliformis* could be detected were phlebotomi. No cutaneous lesions were induced in monkeys injected with the crushed insects, but in the case of four different lots of phlebotomi the blood of the animals so injected yielded cultures of *Bartonella bacilliformis* which produced typical verrucous lesions on inoculation into other monkeys.

The morphology and cultural characteristics of the Bartonella strains obtained from phlebotomi proved identical with those of strains

isolated from human blood and skin lesions. Monkeys which had recovered from infection with the phlebotomus strains resisted inoculation with a human strain of *Bartonella bacilliformis*, and, conversely, monkeys which had passed through an infection induced by the human strain resisted inoculation with the strains obtained from phlebotomi.

The experimental observations described in this paper lead us to conclude that certain phlebotomi act as insect vectors of Oroya fever and verruga peruana. The phlebotomi which have been shown quite certainly to carry the *Bartonella bacilliformis* are those of the species *Phlebotomus noguchii*. *Phlebotomus verrucarum* is also probably a vector, while *Phlebotomus peruensis* remains doubtful in this respect.

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EXPLANATION OF PLATES.

PLATE 45.

FIG. 1. Cutaneous lesions induced in *M. rhesus* I-15 by Phlebotomus Strain 1. Photograph taken 20 days after inoculation. One intradermal nodule had been excised 2 days previously.

FIG. 2. The appearance of the lesions in *M. rhesus* I-1, a Strain 1 first passage animal, 18 days after inoculation. The sacrificed area already shows characteristic minute nodules. All the lesions reached considerable size (Figs. 6 and 7).

FIG. 3. Strain 2. Early culture lesions (two weeks after inoculation) in *M. rhesus* I-23.

FIG. 4. *M. rhesus* I-44, 21 days after inoculation with cultures of Strain 3 from Lots 39 and 45 of *Ph. noguchii*.

FIG. 5. *M. rhesus* I-58, 16 days after inoculation with Strain 4 phlebotomus cultures. The eruption was more general and the edema extensive in this animal. Death occurred 3 days after the photograph was made.

PLATE 46.

FIGS. 6 AND 7. Late lesions in *M. rhesus* I-1 (Strain 1) as they appeared 31 days after inoculation. The most pronounced lesion occurred at the scarification site (center).

FIG. 8. *M. rhesus* I-19, 29 days after inoculation in the same way and at the same time as *M. rhesus* 1.

FIG. 9. *M. rhesus* I-44, 21 days after inoculation with cultures of *Phlebotomus noguchii* Strain 3.

PLATE 47.

Magnification $\times 1,000$.

FIG. 10. Phlebotomus Strain 1, from Lots 1, 2, 9, 14. Gram's stain, counterstained with saturated alcoholic solution of fuchsin.

FIG. 11. Same, stained for flagella, by a combination of Zettnow's mordant and Fontana's ammoniac silver solution.

FIG. 12. Phlebotomus Strain 2, from Lot 20. Gram's stain, counterstained with fuchsin.

FIG. 13. Same, Zettnow-Fontana flagella stain.

FIG. 14. Phlebotomus Strain 3, from Lots 39 and 45. Gram's stain, counterstained with fuchsin.

FIG. 15. Same, Zettnow-Fontana flagella stain.

FIG. 16. Phlebotomus Strain 4, from Lot 54. Gram's stain, counterstained with fuchsin.

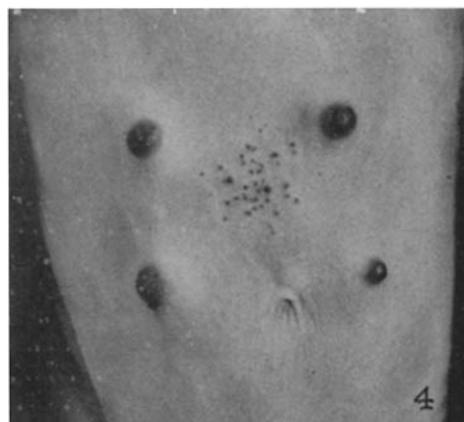
FIG. 17. Same, Zettnow-Fontana flagella stain.



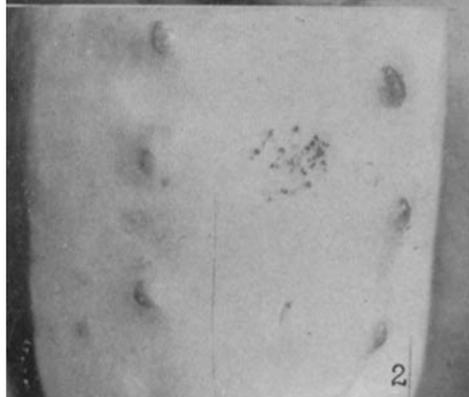
M. rhesus I-15. Strain 1.
20 days after inoculation.



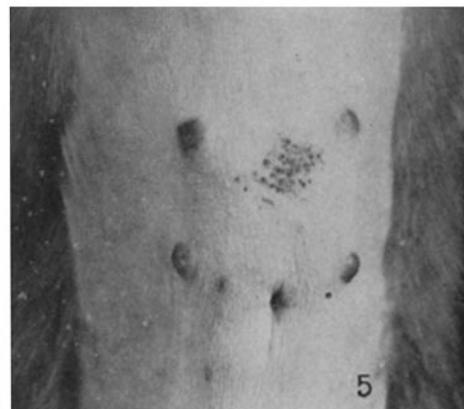
M. rhesus I-23. Strain 2.
14 days after inoculation.



M. rhesus I-44. Strain 3.
21 days after inoculation.



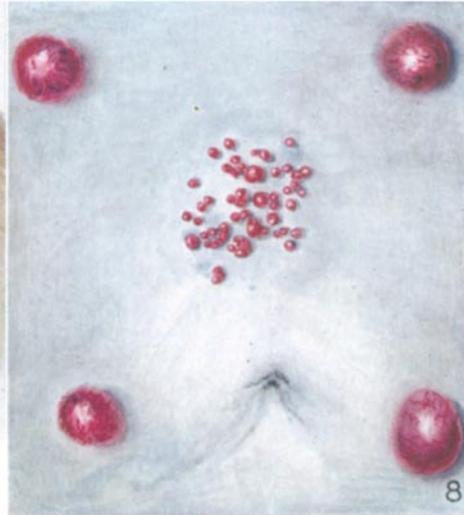
M. rhesus I-1. Strain 1.
18 days after inoculation.



M. rhesus I-58. Strain 4.
16 days after inoculation.



M. rhesus I-1, Strain 1, 31 days after inoculation.



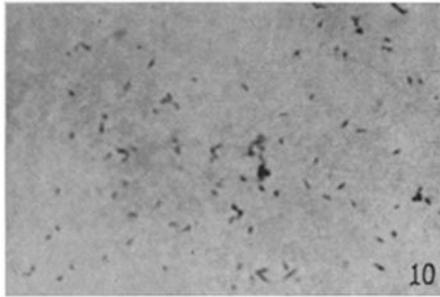
M. rhesus I-19, Strain 1, 29 days after inoculation. Scarified area in center.



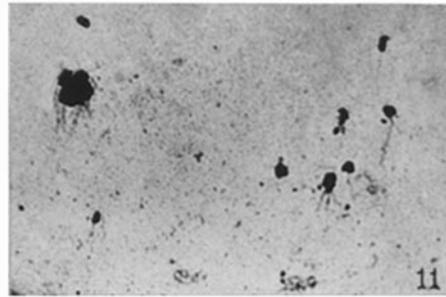
M. rhesus I-1, Strain 1, 31 days after inoculation. The largest lesion arose on the area inoculated by scarification (center).



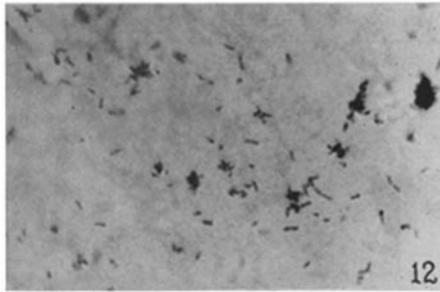
M. rhesus I-44, Strain 3, 21 days after inoculation. Scarified area in center.



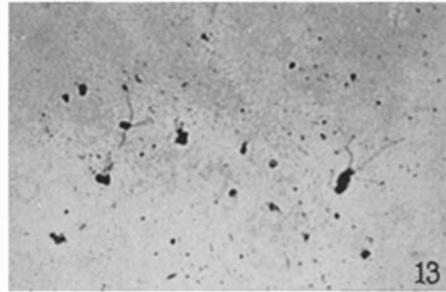
Strain 1.



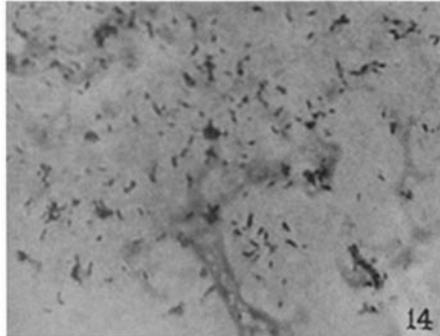
Strain 1.



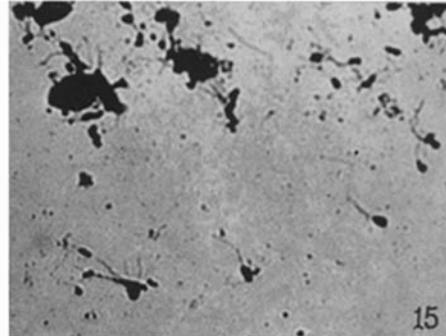
Strain 2.



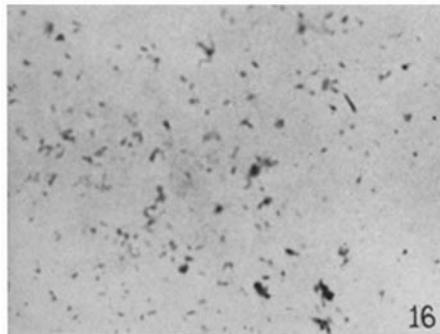
Strain 2.



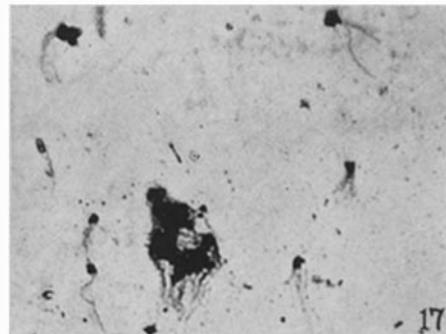
Strain 3.



Strain 3.



Strain 4.



Strain 4.

Gram, counterstained with fuchsin.

Zettnow-Fontana combination stain.

× 1,000.

(Noguchi *et al.*: Etiology of Oroya fever. XIV.)