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## ETIOLOGY OF OROYA FEVER.

### VII. THE RESPONSE OF THE SKIN OF MACACUS RHEBUS AND ANTHROPOID APES TO INOCULATION WITH *BARTONELLA BACILLIFORMIS*.

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In animals experimentally infected with *Bartonella bacilliformis*,<sup>1-4</sup> spontaneous involvement of the skin is far less frequent, and rarely so extensive, as in human verruga. In man the organism probably enters the body through the skin, and, after a preliminary multiplication elsewhere—perhaps in the lymphatic glands—invades the skin from within, giving rise to a persistent endothelial hyperplasia. In animals, when *Bartonella bacilliformis* is introduced locally, a lesion arises which may in time assume dimensions as great as the human verruga nodule. But notwithstanding the persistence of the lesion for many months, and the fact that the surrounding skin is frequently bathed in blood resulting from mechanical injury to the lesion, no new nodules develop. Hence it becomes of interest to determine whether the resistance of the skin in monkeys is due to an anergic condition, such as exists in syphilis, or to inability of the parasite to invade the intact skin.

Two other questions presented themselves in this connection, (1) whether it would be possible to induce verruga formation by injuring an area of normal skin in an animal having active skin nodules elsewhere and carrying *Bartonella bacilliformis* in the blood, and (2) whether autoinoculation would be successful in actively infected

<sup>1</sup> Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

<sup>2</sup> Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

<sup>3</sup> Noguchi, H., *J. Exp. Med.*, 1926, xliv, 715.

<sup>4</sup> Noguchi, H., *J. Exp. Med.*, 1926, xliv, 729.

animals, *i.e.*, whether skin immunity develops during the course of infection, and if so, at what time.

*Resistance of the Intact Skin to Bartonella bacilliformis.*

The question whether *Bartonella bacilliformis* is capable of inducing lesions when applied to the surface of the intact skin is one of practical importance. To test the point, we applied to intact areas of skin whatever material was being inoculated elsewhere. The infectious material was rubbed on two separate areas, each several cm. square, and usually near the sites of inoculation by scarification or intradermal injection. No cutaneous lesions were ever induced by this method, though lesions invariably developed at the sites of the intradermal injections and in many instances also on the areas inoculated by scarification.

Although unusually extensive lesions (*verruca mular*) are often produced by application of infectious material to scarified areas of the skin, there have been many instances in which this mode of inoculation failed, while typical lesions (*verruca nodular*) always arose at the sites of intradermal injection. Hence it is desirable always to use both methods of inoculation.

Chimpanzee,<sup>3</sup> *Pan leucoprymnus*, about 4 years old. *Jan. 19, 1926*, abdominal skin on the right side was shaved and two areas, each about 3 × 4 cm. square, were scarified and smeared with a saline suspension of the skin nodule\* of *M. rhesus* 3. At the same time two adjacent unscarified areas were rubbed with the same suspension. *Mar. 1, 1926*, pinkish, raised indurated lesions had become noticeable on both scarified areas. *Bartonella bacilliformis* was demonstrated in the lesions, both in sections and by culture. No lesions developed on the areas which were not scarified before being smeared with the suspension.

Ourang-utan,<sup>3</sup> *Pongo pygæmus*, about 3 years old. *Apr. 17, 1926*, abdominal skin shaved and an area (2 × 3 cm.) on the right lower quadrant scarified and rubbed with a piece of nodule from the eyebrow of *M. rhesus* 23. An unscarified area on the upper quadrant was vigorously rubbed with the same material. *May 20, 1926*, pinkish, raised, linear lesions, rather pale, have been present on the scarified area for some time. *Bartonella bacilliformis* was demonstrated in the lesions both by culture and in sections. No lesions developed on the unscarified smeared area.

*M. rhesus* 18.<sup>2</sup> *Feb. 15, 1926*, right eyebrow and skin of right abdomen shaved and scarified and a saline suspension of the nodule of *M. rhesus* 5 applied. Adja-

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\* Nodules were removed under ether anesthesia.

cent unscarified areas were rubbed with the same material. Extensive nodules of deep red color developed on the scarified area of the eyebrow within a few weeks and numerous punctiform red miliary nodules on the scarified abdominal skin. The abdominal lesions disappeared within a month, the nodules on the eyebrow remained until the middle of June, when they became small, pale, and fibrous. *Bartonella bacilliformis* was demonstrated in the lesions both by culture and in sections. No lesions developed on the unscarified smeared areas.

*M. rhesus* 23.<sup>5</sup> Mar. 9, 1926, left eyebrow shaved and a scarified area smeared with a piece of the nodule from the eyebrow of *M. rhesus* 18. An adjacent unscarified area was vigorously rubbed with the same piece of tissue. Within 2 to 3 weeks large protruding lesions had appeared on the scarified area, and these progressed to considerable size during the following weeks. *Bartonella bacilliformis* was found in the sections of the lesion. No lesion appeared outside of the scarified area.

#### *Relation of Injuries to Localization of Lesions.*

Whether or not mechanical factors—friction, exposure to minor injury—are involved in the spread of the skin lesions in human verruga is not known. In animals these devices appear to have no influence, as shown by repeated failure to induce localized lesions in infected animals by scarifying the skin or introducing foreign substances (agar, culture medium) intradermally during the height of the infection, when *Bartonella bacilliformis* was demonstrable in the blood. In one unusually susceptible animal, *M. rhesus* 25,<sup>2</sup> in which spontaneous miliary nodules developed, scarification of the abdominal skin had no influence on the course of the general eruption. Similar attempts to induce nodule formation failed also in the chimpanzee, the ourang-utan, and in *M. rhesus* 18,<sup>2</sup> which had at the sites of scarification on the eyebrow one of the most extensive local lesions (*verruca mular*) observed in the course of the experimental work with *Bartonella bacilliformis*.

It is evident that in experimental animals *Bartonella bacilliformis* cannot be made to localize in an area of skin injured either by scarification or by intradermal inoculation of foreign substances, though present in the circulating blood and in the lymph channels, as well as in the skin lesions. Injuries of the skin are not equivalent to the deposition of concentrated infectious material on the scarified skin or in the cutaneous tissues.

<sup>5</sup> Noguchi, H., *J. Exp. Med.*, 1927, xlv, 437.

*Autoinoculability and Superinfection.*

In a disease like verruga peruana, which persists over a long period, there must be a steady process of self-infection. The manner in which the cutaneous lesions spread has not been definitely determined, but it is certain that the skin must retain its susceptibility during periods of remission and relapse. The skin of *Macacus rhesus* and that of the apes is relatively so insusceptible to infection with *Bartonella bacilliformis* that spontaneous eruptions (*verruca miliar*) seldom occur, but it has been possible to induce well marked nodular lesions (*verruca nodular*) in actively infected animals by intradermal inoculation of suspensions of nodular tissue either from the same or from another animal. Such nodules never attained the size of the primary lesions, and they usually began to recede earlier. In no instance was it possible to induce any lesions on scarified areas during the course of infection, though the materials employed induced typical lesions in control animals.

In infections with the strain of *Bartonella bacilliformis* derived from Oroya fever, the existence of active lesions considerably reduced the susceptibility of the skin to subsequent inoculation. Moreover, once the animals were free from *Bartonella bacilliformis*, they were completely refractory to reinoculation, hence the reduction in susceptibility during the course of illness is due to a partial immunity, not to a state of anergy.

On the other hand, in infections with the strain of *Bartonella bacilliformis* from a case of verruga,<sup>6</sup> there have been exceptional instances in which a preexisting infection did not prevent the development of the most severe type of local infection after reinoculation with the same (verruca) strain. *M. rhesus* 33 and *M. rhesus* 34 showed this type of reaction.

*M. rhesus* 33 was inoculated on Apr. 7, 1926, with the saline suspension of a human verruga nodule (Case P 5). Blood taken on Apr. 16 and again on May 27 yielded (in 1:10 dilution) cultures of *Bartonella bacilliformis*.

On June 3 the animal was inoculated intradermally and by scarification with the suspension of a nodule from the eyebrow of *M. rhesus* 41 (second generation of the same strain). On June 24 the lesions had already developed at the sites of inoculation, and by July 1 they were very large and active.

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<sup>6</sup> Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

*M. rhesus* 34 was inoculated on Apr. 10, 1926, with the saline suspension of the nodule of Case P 5. Cultures was obtained from diluted (1:10) blood on April 28, May 12, and May 27. By June 29 a large nodule had developed on the tail.

On June 3 the animal was inoculated intradermally and by scarification with the suspension of the eyebrow nodule of *M. rhesus* 41. *Bartonella bacilliformis* was cultivated from a 1:100 dilution of blood taken on June 20. Large lesions had appeared at the sites of inoculation at this time, and by July 1 the lesions had become unusually extensive.

As these instances show, it is not possible to make any general statement with regard to the partial immunity observed in most animals when passing through a protracted course of infection with *Bartonella bacilliformis*. Immunity may develop so slowly in some animals as not to interfere with the course of a superinfection.

#### *Pathogenesis of the Skin Lesions.*

The verruga-like skin lesion produced by *Bartonella bacilliformis* in anthropoid apes and *M. rhesus* is a slowly progressing infectious angioendothelioma and owes its origin to a delicate reciprocal relation between the endothelial cell and the microorganism. *Bartonella bacilliformis* finds within the cytoplasm of endothelial cells most favorable conditions for its multiplicative phase, and once lodged there multiplies steadily, though perhaps rather slowly, eliciting certain reactions on the part of the infected cells. Since these parasitized cells remain alive for a long time and undergo active mitosis, it is clear that the parasite furnishes a mild type of stimulus which leads to continuous proliferation of the cells. The lesion thus started continues to grow until local as well as general immunity intervenes to terminate the peculiar association of cells and parasite.

The evolution of the verruga lesion in experimental animals may be divided into (1) the incubation period, (2) the initial stage, (3) the mature vascular stage, and (4) the regression. These divisions are of course arbitrary, one stage passing into the next imperceptibly, yet each at its height has its special feature.

*Incubation Period.*—No instance has been observed in which the lesion was macroscopically recognizable within less than 10 days after inoculation. Usually a trace of induration can be detected after 10 to 14 days, though in some cases the incubation period is as long as 3 weeks.

*Initial Stage.*—When the nodular lesion, which becomes noticeable as a slightly raised area, 1 to 2 mm. in diameter, has reached a size of 4 to 6 mm. and protrudes 2 to 3 mm. beyond the surrounding normal skin, the color becomes slightly pinkish, and edema of the surrounding tissues becomes evident. The nodule is now firm and well defined to the touch and can be easily separated from the overlying skin and the loose connective tissue in which it is embedded. The cut section is grayish pink, edematous, but not soft, and microscopically the nodule is seen to consist of masses of endothelial cells, placed close together, lying among capillary vessels and connective tissue fibers. A small number of lymphocytes and polymorphonuclear leucocytes are seen in the interstices of the cutis, but not within the nodule itself. The endothelial walls of the capillaries are thickened, and the lumina of the blood vessels narrowed. Practically every endothelial cell in the nodule contains *Bartonella bacilliformis*, some cells being packed with clumps of organisms. The endothelial cells lining the capillaries are often filled with similar masses of microorganisms. The histological picture at this stage is very simple.

*Mature and Vascular Stage.*—The interval from the preceding stage to this one varies from a few to 10 to 14 days and persists for 10 days to 2 weeks.

The increase in size of the nodule may be gradual or even relatively rapid until the lesion measures 8 to 10 mm. or more in diameter. The color first becomes bright, then deep, red. The overlying skin is shiny, and the epidermis scaly in places. The nodule is still firm and is sharply demarcated from the surrounding tissues. The color may change to a dark bluish red, suggesting that of a cherry. This type of lesion, especially when deep seated and less vascular on the surface, is characteristic of the form of human lesion known as *verruca nodular*. Occasionally the overlying skin yields to the increasing pressure from within, and the lesion becomes pedunculated.

The lesions found on the scarified lines of the skin are seldom round; on the contrary, they are usually irregular at the surface, and the mass resembles a raspberry in form and color, being made up of a number of small, translucent, red, drupelet-like nodules. The entire region adjacent to the lesion is edematous. The lesion is devoid of an epidermic layer, hence it bleeds readily on slight friction. This type of lesion corresponds to that termed in man *verruca mular*.

In a few animals spontaneous nodules have appeared on the skin at sites remote from those inoculated; these correspond to the general eruption in human cases known as *verruca miliar*. Such lesions have always been small, none becoming larger than 1 to 2 mm. in diameter, but their clinical course is similar to that of the nodules induced by intradermal inoculation or by scarification.

On microscopic examination, nodules or lesions removed during the mature and vascular stage present a somewhat more complex picture than those of the initial stage. The large number of capillary vessels intersecting dense masses of closely packed endothelial cells and the increase of fibrous connective tissue irregularly penetrating the nodular structure are striking features of the lesions. Numerous fibroblasts and fibrils lie between islands of angioblasts, the appearance somewhat suggesting that of a fibrosarcoma. In places there is hyperplasia of the epidermis, and some large detached masses of epithelioid cells find their way deep into the nodular zone, a circumstance which complicates the interpretation of the cellular elements composing the nodule. Some of these invading epithelioid cells show hydropic degeneration. Degenerating endothelial cells and numerous erythrocytes are present in some places and are taken up by migrating macrophages, which also engulf the dead polymorphonuclear leucocytes simultaneously present in these areas. Along the interstitial spaces penetrated by connective tissue fibers, plasma cells and mast cells are sometimes rather numerous. The lumina of the capillary vessels are often occluded or compressed by proliferating endothelial cells. Mitotic figures are common. The skin may be adherent.

The microorganisms are more uniformly present in the endothelial cells along the periphery of the nodule than among the cells in the older foci. None except endothelial cells contain *Bartonella bacilliformis* in the cytoplasm, and no extracellular localization of the parasites can be detected.

When a secondary bacterial invasion has taken place, there are found, needless to say, an enormous number of polymorphonuclear leucocytes loaded with the contaminating bacilli or cocci. At times these bacteria-carrying leucocytes are taken up by endothelial cells and give rise to a confusing appearance, as though the secondary invaders were the primary cause of the hyperplasia.



*Regression.*—The time of beginning of the regressive process depends on the individual animal's resistance to the infection, and the process of regression is intimately associated with the acquired immunity, local or constitutional. Early regression, before full development of the lesion, has been rather frequent in the experimental animals. Once the nodule has fully developed, however, considerable time is required for its complete resolution, 4 to 5 months in some instances. The first indication of retrogression is the gradual loss of the deep red color. As the color fades the size of the nodule begins to diminish, the paling and diminution continuing until finally only a tiny colorless wart remains. When the nodule ruptures spontaneously at the beginning of the period of regression, the whole mass sloughs and the wound heals rapidly with scar formation. The result is quite different, however, when the nodule is injured, or partially removed by surgical operation during its developing stages; in this instance the lesion acquires renewed vigor and spreads far beyond its previous limits in the form of a *verruca mular*; it becomes malignant.

The histological appearances of the lesions, as just presented, are similar to those observed by the Harvard Commission<sup>7</sup> in human and experimental verruga tissues, of which they carefully studied a large number. The eosin-staining inclusions so constantly encountered in the endothelial cells, both in human and monkey nodules, and particularly emphasized by the Commission, are of considerable significance. These reddish granules, which may have been identical with those described by Mayer, Rocha-Lima, and Werner<sup>8</sup> as Chlamydozoa, in all probability represent intracellular forms of *Bartonella bacilliformis*, somewhat less distinctly stained than in our preparations. The possibility of indistinct staining is one which cannot be ruled out in connection with this difficultly staining microorganism.

<sup>7</sup> Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaturú, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

<sup>8</sup> Mayer, M., Rocha-Lima, H., and Werner, H., *Münch. med. Woch.*, 1913, lx, 739.

## SUMMARY.

*Bartonella bacilliformis* failed to induce lesions when merely rubbed on the surface of the intact skin of a chimpanzee, an ourang-utan, and numerous *Macacus rhesus* monkeys, although when applied to the scarified skin of the same animals it gave rise to extensive lesions.

Application of infectious material to the scarified skin did not always induce verruga lesions, but intradermal inoculation almost invariably gave rise to nodule formation.

The localization of *Bartonella bacilliformis* in the skin is not, in experimental animals, determined by mechanical factors, since scarification of the skin or intradermal injection of foreign substances in monkeys infected with *Bartonella bacilliformis* does not give rise to verruga formation.

The degree of susceptibility of the skin tissues appears to be considerably diminished during the course of experimental infection with *Bartonella bacilliformis*. Inoculation of the scarified skin of infected animals gave uniformly negative results, and intradermal inoculation induced only a mild local reaction. In a few exceptional instances, however, of animals previously infected with the strain of *Bartonella bacilliformis* derived from a human verruga nodule, reinoculation with the same strain gave rise to unusually marked reactions.

The evolution of the skin lesion induced in experimental animals by *Bartonella bacilliformis* may be divided into four stages, the period of incubation, the initial stage, the mature and vascular stage, and the regression. In the initial stage the lesion is a pure angioendothelioma, but in the stage of full development the histological picture is complicated by connective tissue proliferation and occasionally also by penetration of epidermis into the lesion. The demonstration of *Bartonella bacilliformis* in the endothelial cells distinguishes the lesion from others which simulate it.

The cutaneous lesions known as *verruca nodular*, *verruca mular*, and *verruca miliar* have been reproduced in monkeys.