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## ETIOLOGY OF OROYA FEVER

### XVI. VERRUGA IN THE DOG AND THE DONKEY

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PLATES 21 TO 23

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In the older literature on verruga<sup>1,2,3,4</sup> are found statements that in endemic centers the disease may sometimes occur spontaneously in domestic animals, particularly horses, mules, dogs, and pigs. That distinct differences in the appearance of the nodular lesions existed, or were thought to exist, among these animals, is apparent from the fact that they were used as criteria for describing certain types of lesions in man; for example, the deep-seated, bulky lesions were, and still are, called "mular" or "mulaire," whereas the superficial ones were sometimes referred to as "verrugas de caballo" (verruca of horses). The variation in appearance of the nodules seems to have been accepted by tradition, since careful descriptions of verruga in animals cannot be found, and actual records of experimental inoculations in the closely related species of horses, mules, and donkeys are apparently very rare. The experiment of Ribeyro, Mackehenie, and Arce,<sup>5</sup> in 1913, with a donkey, is the only one of the kind on record. The animal was inoculated by scarification of the skin at the inner canthus of the eye, and on the nose, with the "pulp" of a verruga nodule obtained from a patient. After 21 days the skin at the sites of inoculation showed papules 3 to 4 mm. high, which increased only very slightly in size during the next 6 days. Then they began to regress and after 11 days had entirely disappeared. The reaction seems to have been a very mild local one.

In their attempts to find an animal susceptible to *Bartonella bacilliformis*, Noguchi and Battistini<sup>6</sup> tried ringtail, *rhesus*, Java, and green monkeys, dogs, rabbits, mice, rats, and guinea pigs, and found that

results were definite only in the *rhesus* monkey. Later, experiments were started (Noguchi) in dogs, horses, and donkeys, the results of which are recorded below. In the dog mild verruga was induced, in the horse and in burros the results were negative so far as concerned the production of true verrugous skin lesions; in the horse and in one burro there was induration at the sites of inoculation, which lasted 3 to 4 weeks, but *Bartonella bacilliformis* was not recovered from the lesions. Further experiments, with donkeys, have been more successful.

Dog 1, female, inoculated March 23, 1926, intradermally, on eyebrows and abdomen, with (a) a mixture of 4-weeks old cultures, 1st and 2nd generations, grown on blood agar slants and leptospira medium, from the blood of *M. rhesus* 14,<sup>7</sup> a chimpanzee,<sup>7</sup> and *M. rhesus* 18,<sup>8</sup> and (b) a saline suspension of nodular tissue excised\* from *M. rhesus* 18. 2 cc. of the culture mixture were injected intravenously. Small nodules developed after 8 days at the sites of culture inoculation, but none at the sites inoculated with nodule suspension. Four days later one of the nodules had reached a diameter of 0.5 cm. and was excised.\* *Bartonella bacilliformis* was recovered in culture from a suspension of the tissue. Blood cultures made at this time were negative.

*Microscopic examination of nodular tissue:* In the spaces among the thick collagen fibers of the dermis there is a proliferation of endothelial cells. This process is chiefly diffuse, but small foci also are found. There is also a considerable polynuclear exudate and a moderately extensive plasma cell exudate. Except for the latter findings, the lesion is histologically similar to the lesions produced in monkeys by *Bartonella bacilliformis*.

Colt, about 3 years old. Inoculated March 30, 1926, intradermally, at two sites on the left flank, with mixed cultures (from blood agar slants and semisolid leptospira medium) of *Bartonella bacilliformis*. There was no change in the animal's temperature, which was normally 100.4°F. (38°C.), and blood culture was negative 8 days after inoculation. After 20 days there was definite induration at the sites of inoculation, which disappeared within the next two weeks. A second inoculation was made at 4 sites on the neck, on April 22, with the material inoculated on that day into Donkey 1. After 4 to 6 days there was marked induration at the two sites of culture inoculation, but none at the sites inoculated with nodular tissue and blood, and there was no fever.

Donkey 1. Inoculated April 22, 1926, at 4 sites on the left flank and 3 on the left side of the neck, with (a) 15-day culture from blood agar slant, 5th generation from *M. rhesus* 7,<sup>8</sup> (b) 14-day culture on leptospira medium, 1st generation from *M. rhesus* 29,<sup>9</sup> and (c) saline suspension of nodular tissue from *M. rhesus* 18,<sup>8</sup>

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\* All operations were performed under ether anesthesia.

and (d) citrated blood from *M. rhesus* 25.<sup>8</sup> 8 cc. of the mixture were injected into the jugular vein. There was no change in the animal's temperature, which remained at about 100°F., and blood culture was negative 4 days after inoculation. At this time there was marked edema along the neck and flank and at other points remote from the sites of inoculation. After 8 days there was definite induration at the inoculated sites, and one of the "nodules" was removed for culture and section. *Bartonella bacilliformis* was not recovered from the suspension of tissue, nor were the organisms seen in the sections. Histologically, however, the section has the appearance of that from a very early verruga lesion. Several minute foci of endothelial cell proliferation are present in the deep portions of the dermis, and there is a tendency to the formation of sheets of cells and capillaries. A moderate polynuclear infiltration is also present.

In the second donkey the inoculation gave rise to edematous areas remote from the areas of inoculation, but there was very little reaction at the inoculated sites.

Donkey 2, inoculated Nov. 30, 1926, intradermally, on the left side of the neck, and also intravenously (4 cc.), with 8-day cultures of *Bartonella bacilliformis* grown on blood agar plates. There was no change in the animal's temperature, which remained slightly below or above 38°C. Rather large, fluctuating swellings appeared within 48 hours on various parts of the body, one on the side of the back, and others on the flank and abdomen. Blood culture was negative 72 hours after inoculation, and also after 20 days. The swellings were still present 2½ months later, and one was punctured; the aspirated fluid was sterile. A piece of tissue, removed from another "nodule" at this time for section, was histologically negative. Six weeks later a saline suspension of tissue from one of the "nodules" was inoculated into *M. rhesus* 30, with negative results, both as to blood culture and local lesions. The animal's appearance was nearly normal 284 days after inoculation. Its weight at this time was 440 lbs. Reinjection of the animal 8 months later, that is, a year and a half after the original inoculation, with live cultures, produced the same reaction as before, and in this instance there were generalized small scaly eruptions all over the body, which persisted about a week.

The results in Donkey 2 were difficult to interpret, and two more donkeys were inoculated, one with killed cultures of *Bartonella bacilliformis*, and the other with a filtrate of living cultures. The reaction in these two animals was much the same as in Donkey 2. In the animal receiving the filtrate (Donkey 3) the swellings appeared within 72 hours after inoculation (Fig. 1); in Donkey 4, inoculated with heat-killed cultures, they did not develop fully until about 10 days after inoculation. In both, however, they persisted for several weeks.

The study of sections made from the swollen areas yielded no explanation of the phenomenon which occurred in these two animals.

Donkey 3, weight about 500 lbs., injected April 4, 1928, intravenously, with filtrate (Berkefeld V) of 14-day cultures of *B. bacilliformis*. The temperature was 102.4°F. late in the day of inoculation, but thereafter remained a few tenths of a degree below or above 100°. Large raised areas developed in 72 hours, as in Donkey 2 (Fig. 1). Blood culture was negative 9 days after inoculation. Control *rhesus* monkeys (injected with the same material, intravenously and intradermally) developed no skin lesions, and blood cultures were negative. Cultures of the inoculated material also proved its sterility.

Donkey 4, 650 lbs., injected April 16, 1928, intravenously, with cultures of *Bartonella bacilliformis* killed by heating 30 minutes at 60°C. This material was proved sterile by culture tests and animal inoculation. The animal's hide was not perfectly smooth, and the natural prominences were outlined with a skin pencil before inoculation. 10 days after inoculation this animal was found to have developed the same swellings as Donkeys 2 and 3, but they were less marked. The appearance of the animal 25 days after inoculation was similar to that of Donkey 3. A piece of tissue removed from a swelling on the left side of the animal 8 months after inoculation was found on microscopic examination to consist solely of fat tissue.

The donkeys used in the first four experiments were of the small (440–650 lbs.) light gray variety commonly known as the “burro.” The fifth donkey, in which definite verruga lesions were produced (Fig. 3), was a larger animal (800 lbs.) and brown in color. So far as can be ascertained, however, the species is the same.

Donkey 5. Weight 800 lbs. Five intradermal injections were made, on Jan. 5, 1929, with a mixture of living cultures of the 5 strains of *Bartonella bacilliformis* on hand in the laboratory,<sup>10,11</sup> two into the skin of the left flank, two on the right ear, and one on the left ear. 10 cc. of the same mixture were injected intravenously. The material produced, on injection into the skin of the ears, swellings about 1 cm. in diameter, which did not subside but slowly increased in size, so that after 6 days they were 2 cm. in diameter and 12 mm. high (Fig. 3). They were dome-shaped and slightly fluctuant. The skin over them remained intact and was of the normal black color of the donkey's ear. The sites of inoculation on the flank secreted a clear serous liquid for a few days, without papule or nodule formation, and then completely healed.

The animal continued to receive intravenous injections of mixed strains of living cultures of *Bartonella bacilliformis*, 20–30 cc. at each dose, twice a week for 14 weeks, the idea being to produce, if possible, a strong local and general reaction, in the hope of obtaining an immune serum for use in other experiments.

After the nodules on the ears had reached a diameter of 2 cm. they remained stationary for a period of 3 weeks, after which they gradually became smaller until at 72 days after inoculation they were invisible. Blood cultures were made repeatedly throughout the period of observation and yielded uniformly negative results.

One of the nodules on the right ear was aspirated on the 22nd day after inoculation and slightly less than 1 cc. of turbid liquid obtained which proved by culture tests to be sterile. Both nodules on the right ear were punctured three days later, but no liquid was obtained from either. The nodule on the left ear, similar in all respects to those on the right ear, was then removed under novocaine anesthesia. A portion was used for inoculation of a *rhesus* monkey, and another portion for histological study.

*Animal passage.* *M. rhesus* D-1 was inoculated intradermally and by scarification on Feb. 16, 1929, with a saline suspension of a part of the nodule removed from Donkey 5. After an incubation period of 9 days typical nodules (Fig. 4) developed at the sites of inoculation, and blood culture made the following day was positive for *Bartonella bacilliformis*. Nineteen days after inoculation the nodules were mature, the area inoculated by scarification showed fully developed lesions, and after 51 days recovery was complete. The disease ran a typical course. The culture of *B. bacilliformis* obtained from the blood of this animal and a subculture from it, on intradermal inoculation into *M. rhesus* D-2 and D-3 produced typical reactions (Figs. 5 and 6).

*Histological examination* (Figs. 7 and 8). The tissue removed from the ear of Donkey 5 consisted of the covering epidermis, a narrow rim of normal skin encircling the nodule, and a superficial portion of the nodule itself. The latter, irregularly marked off from the rest of the tissue, was soft, gray with a faint pink tinge, and about 4 mm. thick at the center, tapering off gradually in thickness toward the edges. The tissue was fixed in Regaud's fluid, and sections were stained with hematoxylin and eosin and with Giemsa's solution.

The hematoxylin and eosin sections, examined with the naked eye, show a thin normal layer of epidermis covering a layer 1 to 1.5 mm. thick of apparently normal dermis. Beneath this is a collection of small round bodies, closely packed and tending here and there to become conglomerate. Each of these individual nodules, varying from 1 to 3 mm. in diameter, stands out from the rest of the tissue by its deeper hematoxylin stain. Together they comprise the nodular tissue proper.

Microscopically the minute nodules which make up the large nodule are in general fairly distinct from one another, but in places they tend to fuse. They are all very densely infiltrated with polynuclear leucocytes, which somewhat obscure the underlying structure by their presence. However, numerous slit-like spaces of variable length are seen within the minute nodules, lined with large, oval, flat cells with clear oval nuclei. These cells resemble endothelial cells. Some of the spaces contain serum; others are empty. With higher magnification

there are seen larger collections of these endothelial cells forming sheets or running in bands, and having a tendency to form blood-containing capillary-like structures.

The overlying epidermis and the narrow zone of dermis, with its numerous hair follicles and sweat glands, are normal. Though there is no distinct capsule, these tissues are separated sharply from the collection of minute nodules beneath. The surgical line of excision passes through the nodular material.

These findings show that the unit of the pathological process situated in the deep portions of the dermis is a minute nodule, from 1 to 3 mm. in diameter. The units may be discrete or tend to fuse. Each is composed of a considerable proliferation of endothelial cells, arranged in sheets, bands, and tubes. Superimposed, there is a very marked polynuclear infiltration, as was found in the section of nodular tissue from Dog 1.

#### SUMMARY AND CONCLUSIONS

In the experiments here reported, definite verruga lesions, in which the presence of *Bartonella bacilliformis* was established by culture or by passage to *rhesus* monkeys, were produced in a dog and in a donkey by inoculation of cultures or monkey passage strains. The reaction induced in these animals was entirely local, however; blood cultures were sterile. Histologically, the lesions produced were similar to those obtained in monkeys by inoculation of *Bartonella bacilliformis*, except for the presence of a marked polynuclear leucocytic exudate.

In another donkey a lesion histologically suggestive of verruga was produced, while in one donkey and a horse the results of inoculation were negative or indefinite.

The intravenous injection of a filtrate or of heat-killed cultures of *Bartonella bacilliformis* into two donkeys was followed by the appearance of large, soft, subcutaneous swellings, on various parts of the body, not resembling in any way verruga lesions.

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EXPLANATION OF PLATES

PLATE 21

FIG. 1. Donkey 3, 72 hours after intravenous injection with a filtrate of cultures of *Bartonella bacilliformis*, showing the large swellings which arose on the side.

FIG. 2. Photograph of the same animal, taken before inoculation. For comparison with Fig. 1.

PLATE 22

FIG. 3. Appearance of nodules on right ear of Donkey 5, 39 days after inoculation. The nodule on the left ear had been partly excised 14 days previously, and the wound was in process of healing.

FIG. 4. *M. rhesus* D-1, showing verruga nodules on abdominal wall 10 days after inoculation with emulsion of nodule from ear of Donkey 5.

FIG. 5. *M. rhesus* D-2, 31 days after inoculation with culture of *Bartonella bacilliformis* from blood of *M. rhesus* D-1.

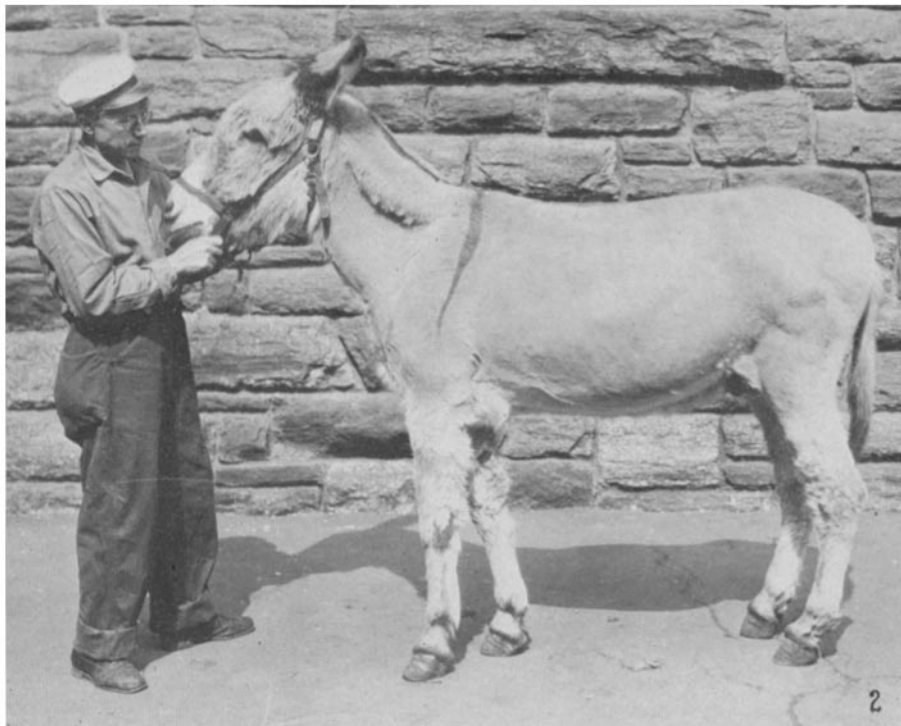
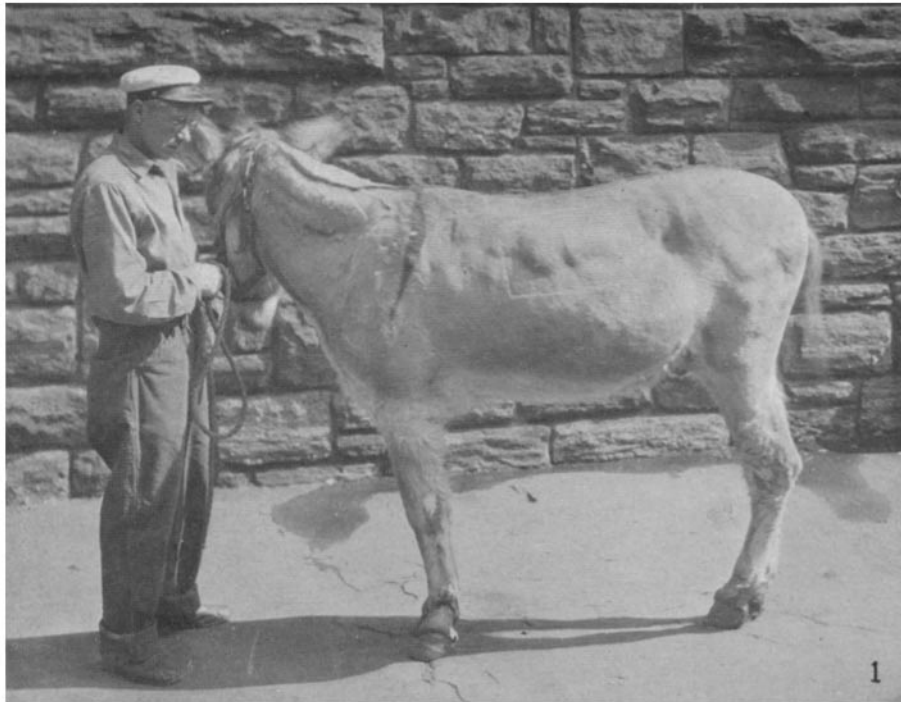
FIG. 6. *M. rhesus* D-3, 22 days after inoculation with subculture of *Bartonella bacilliformis* from blood of *M. rhesus* D-1.

PLATE 23

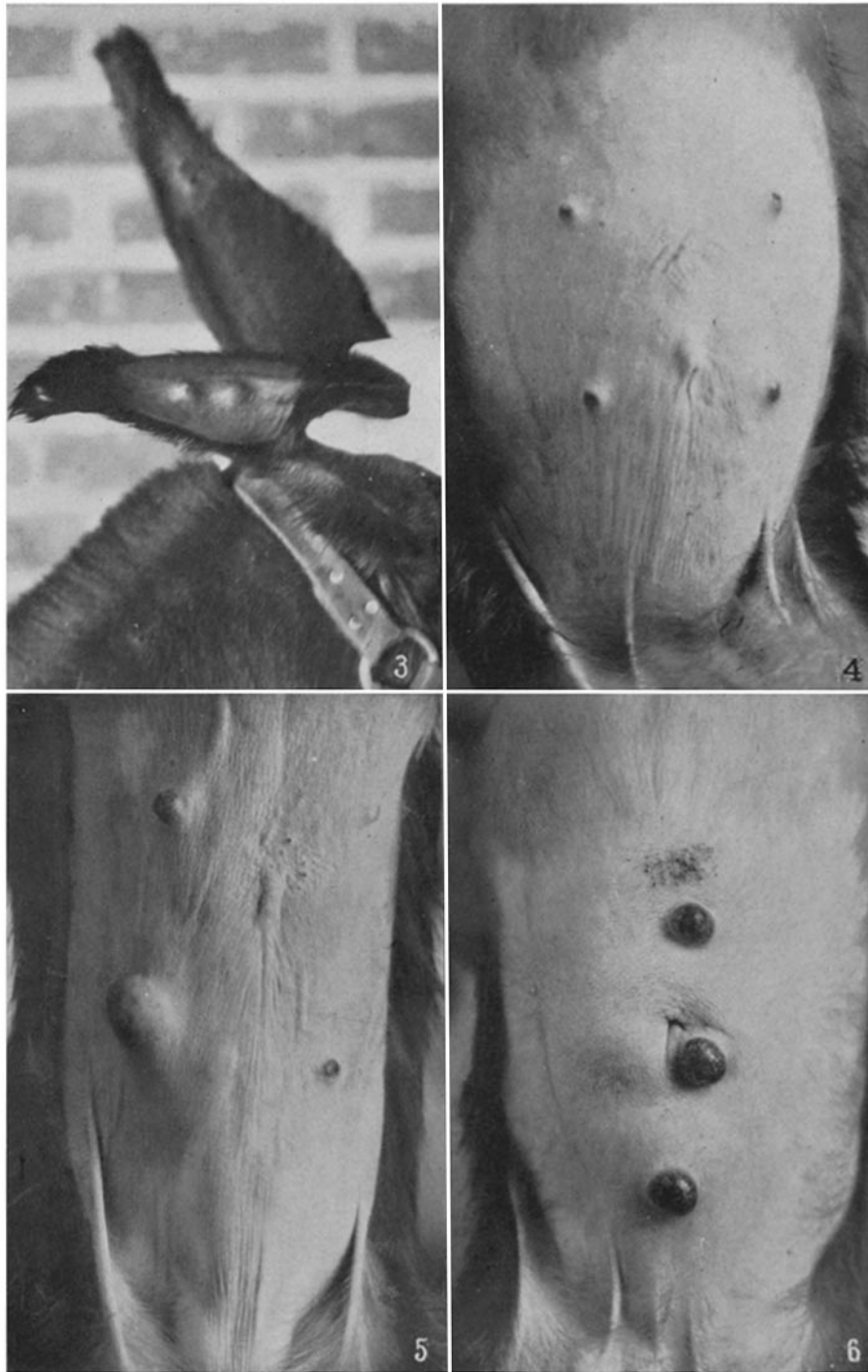
FIG. 7. Low power magnification ( $\times 105$ ) of section from nodule on ear of Donkey 5, showing one unit of the lesion.

FIG. 8. Higher magnification ( $\times 600$ ) of section from same nodule, showing proliferation of endothelial cells, lymph and blood spaces, and polynuclear infiltration.

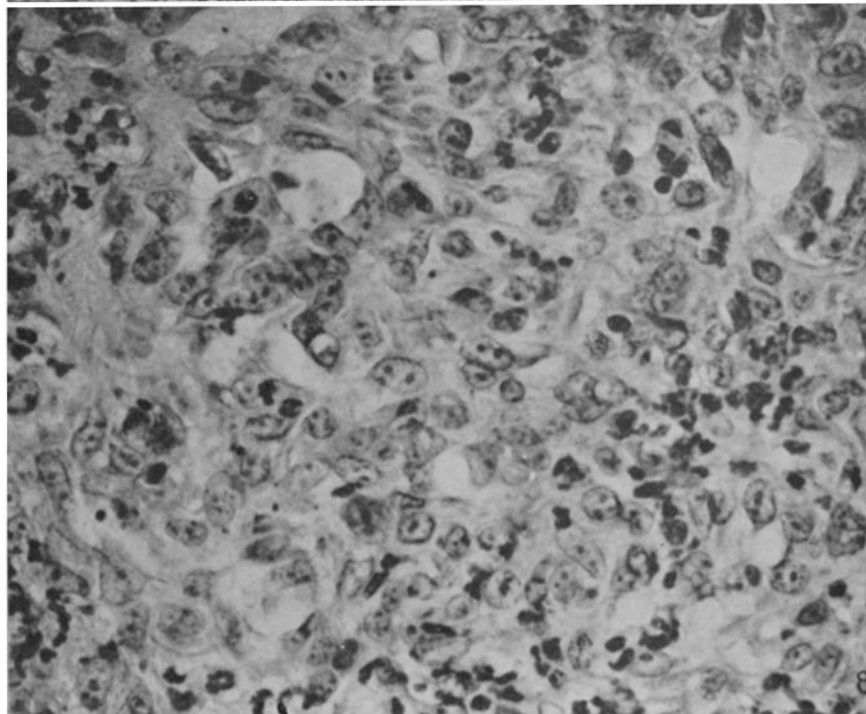
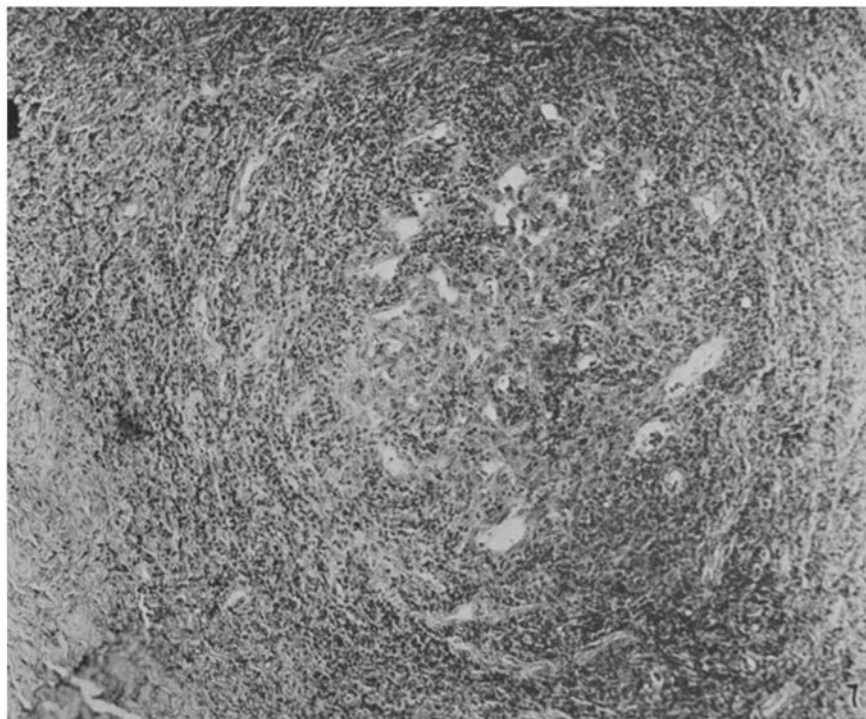




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