A FURTHER NOTE ON INOCULATION OF MONKEYS WITH HUMAN LEPROSY MATERIAL AFTER SPLENECTOMY

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INTRODUCTION

The first report of this work, published in 1939 (1), indicated that inoculation following splenectomy appeared to be the most promising method of infecting monkeys with leprosy. Seven animals were involved in the experiments then reported, but in only one of them was there any evidence of possible dissemination of the infection. It was felt that the reason for failure was the factor of individual variability of resistance, and it was indicated that many monkeys may have to be used before one is found which is susceptible.

Since then Collier (2), working at first in collaboration with Oberdoerffer, has claimed infection of monkeys that were fed on a diet of *Colocasia antiquorum*. He has stated that acid-fast organisms were found in them, and that one of the features of the infection was a widespread erythematous rash. In view of this finding, it is of special interest that in two of our monkeys acid-fast organisms which in some respects resembled *Mycobacterium leprae* were *recovered*, and an erythematous rash was noticed. Both of these monkeys had been fed on colocasia, receiving two tubers a day.

The continuation of this work is reported here, together with certain observations on the lepromin test in inoculated and uninoculated monkeys. The results of inoculations to be presented are those obtained since the time of the previous communication. A total of sixteen monkeys have been inoculated, only three of the original group remaining. Three died within a few days after inoculation and are therefore not included, and four have been inoculated too recently to be considered here. The nine that are discussed can be divided into three series, as follows:

SERIES A. Three monkeys splenectomized and inoculated with leprosy material on several occasions.

SERIES B. Three monkeys splenectomized, inoculated with leprosy material and fed on *Colocasia antiquorum*.

SERIES C. Three monkeys splenectomized and inoculated, with blockage of the reticulo-endothelial system with India ink.

The first two animals of this group were considered in the previous report; the third is a new one.

SERIES A

MONKEY 3.—The last inoculation of this animal previously reported was the third, made on October 24, 1938. Three months later (January 21, 1939) the abdomen was opened and another nodule was fixed to the omentum. No evidence of the previous inoculation was seen. Subsequently the axilliary lymph nodes became enlarged, due to a discharging sinus in the anterior abdominal wall; gland puncture revealed no acid-fast bacilli. A number of discharging sinuses developed around the operation wound, and the smears from these showed acid-fast bacilli at varying times and intervals. All of the wounds healed within six weeks, however, and the acid-fast bacilli disappeared.

On July 24, 1939, the abdomen was opened for the fifth time. All of the previous nodules had disappeared. A fresh one was fixed to the peritoneum. The abdominal wound broke down and discharged acid-fast bacilli, but this healed after a few weeks.

On May 6, 1940, there being little evidence of the last nodules implanted, a sixth inoculation was made, a nodule being fixed to the peritoneum in the usual way. The wound healed without complications. The animal is still under observation.

MONKEY 7.—The last inoculation of this animal reported was made on November 5, 1938; the sinus resulting from it had healed by December 3, 1938. This monkey was left alone except for testing with lepromin until November 18, 1939, when it was again inoculated intraperitoneally. The animal died suddenly three days later after the operation. The postmortem findings were suggestive of death from snake bite, but otherwise nothing abnormal was discovered; there was no evidence of dissemination of the bacilli.

MONKEY 16. — This animal was heavily infected with *Plasmodium knowelsii* artificially, three and one-half months before the experimental inoculation, and recovered spontaneously from that infection. Parasites were not found in subsequent repeated examinations. On February 17, 1940, splenectomy was performed and a nodule was stitched to the splenic stump. Quinine, grs. 2 per day, was given to prevent relapse of malaria. Between then and April 15, an ulcer developed in the neighborhood of the abdominal wound. Smears from the ulcer showed numerous acid-fast bacilli, in clusters and in globus formation. The ulcer subsequently healed. On May 22, the animal was reinoculated, and it is still under observation.

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* The details for all animals have been included in the table prepared for the note following this article. See page 100.

SERIES B

The three animals in this series have been fed on colocasia. From January to May, 1939, two lightly boiled tubers were given each day. From that time to date (January, 1941), unboiled tubers were crushed and given, the animals having to be forcibly fed.

MONKEY 6.—The last inoculation of this animal reported was the second, made on November 28, 1938. After a somewhat critical time the monkey recovered, and by March 27, 1939, the abdominal nodule had become completely absorbed. On May 5 the abdomen was again opened and a nodule was attached to the mesentery and fixed to the abdominal wall. On June 19 both axilliary glands were seen to be enlarged. These were aspirated but no acid-fast bacilli were found.

On October 3, 1939, the monkey developed weakness of the hind limbs, with difficulty in stretching. Six days later an erythematous lesion appeared on the abdomen. Smears showed a few acid-fast and many fungus organisms, including a considerable number of diphtheroids. By October 24 the patch was less obvious, but it did not disappear completely. From this time until the animal died the paralysis of the lower limbs became more apparent. On January 17, 1940, the erythematous patch again became conspicuous and extended to the axilla; the surface was moist, sticky, and foul smelling. A smear showed the same variety of organisms as before. Cultures, both aerobic and anaerobic, were made; besides staphylococci and sarcinae, an anaerobic nonacid-fast bacillus was grown which had the appearance of a diphtheroid. An attempt to transmit this infection to another monkey by scarification was unsuccessful. Betaxin, one ampule daily for ten days, caused no improvement. On February 28 a blood smear was taken and anemia of the pernicious type was diagnosed. The monkey died before treatment could be instituted.

Postmortem examination.—Body extremely pale, with areas denuded of hair, especially over the lumbar area and extremities. Abdominal viscera pale; slight enlargement of the mesenteric glands. There was no evidence of the nodules implanted on three occasions between August, 1938, and May, 1939. Considering the size and the number of the inoculations it was somewhat remarkable that the nodules were so completely absorbed. The splenic stump was reduced to a small fibrous strand. There was no naked-eye evidence of any dissemination of the infection. The main nerve trunks were examined and smears taken.

Bacteriology: Smears taken from the ear-lobes and the skin of the groin showed evidence of infection with the saprophytic organism mentioned. Here and there, however, there appeared a few single acid-fast bacilli of a different appearance, morphologically more like *M. leprae*. No acid-fast bacilli were found in a nasal scraping. In smears from the mesenteric and lumbar glands a few broken acid-fast bacilli were seen, and some larger masses which had the appearance of disintegrated globi. The same appearance was seen in smears from the kidneys and liver, but smears from bone-marrow, testis, and nerves were negative. Sections were made of these organs but no bacilli were found in them.

A guinea pig was inoculated with a suspension of ground-up material from one of the mesenteric glands. The animal showed no sign of tuberculosis during the next three months.

MONKEY 9.—This animal was splenectomized on January 21, 1939, and a nodule was fixed to the splenic stump in the usual way. On March 23 a discharging sinus developed along the operation wound, and smears made of the matter expressed from it showed clusters of acid-fast bacilli and a number of intracellular forms. This sinus persisted for three months and the implanted nodule gradually sloughed out, after which the wound healed.

The animal was reinoculated on July 23. One month later the nodule appeared to be much enlarged and was fixed to the abdominal wall. As there was little change after another two months the animal was subjected to a third inoculation, on October 14. On opening the abdomen the previous nodule was felt and a number of adhesions noted; these were not disturbed. A fresh nodule was fixed to the mesentery. After ten days the wound became septic. As this condition had not cleared up two weeks later, prontosil solution was applied as a packing and this facilitated rapid healing. On November 27, 1939, the nodules implanted in the last two inoculations were felt on abdominal palpation. On December 3 multiple sinuses appeared along the operation wound, and smears from one showed clusters of acid-fast bacilli. By January 17, 1940, all sinuses had healed.

On May 6, 1940, a fourth nodule was implanted. The resulting wound healed slowly. Six weeks later an erythematous patch appeared on the abdominal wall. Scrapings showed diphtheroid organisms similar to those found in monkey 6. Smears from the ears were negative, as were cultures made from the scrapings from the erythematous patch. Simultaneously with the patch on the abdominal wall there was noticed an area on the back denuded of hair. Smears from this area were also negative.

On September 26 a pink fibrous nodule was observed on the anterior abdominal wall. This developed into an erythematous patch. The erythema in the groin disappeared, but the skin of the abdominal patch showed a greenish coloration. Paresis of the legs gradually developed and the monkey could not stretch them. A blood smear showed nothing abnormal. The paralysis increased, the monkey became weaker, and hair began to fall. On October 14 smears from the eyebrows showed a few acid-fast bacilli with the same morphological characteristics as those found previously. Five weeks later the animal was very pale, and erythematous patches appeared again in the groins and on the abdomen, their surfaces moist and sticky. On December 2 the abdomen became bloated and the animal was very short of breath. The blood again showed no signs of pernicious anemia. Death occurred on December 4, 1940.

Postmortem examination.—General apppearance: Abdomen bloated; slight curvature of the spine. Numerous areas denuded of hair on body and limbs, with erythematous, moist, and foul-smelling areas on the abdomen and groins. Abdomen: No fluid in the peritoneal cavity, and nothing of note in the viscera. No trace of the nodules which had been inserted on four occasions, and no apparent lesions of any kind in the mesentery or omentum.

Bacteriology: Smears from the erythematous skin patch on the abdomen showed numerous bacilli, some acid-fast, some partially so, others non-acid-fast. In appearance these organisms were similar to those obtained from monkey 6. The acid-fast forms were scattered among the others and were present in clusters as well as singly. Smears from ear clips and a nasal scraping also contained similar organisms. Smears from the bone

marrow and lymph glands showed a few acid-fast granular rods. Smears from the liver, kidney, lung, spinal cord, and larger nerves were all negative. Lowenstein's and Dorset's media were inoculated, with negative results.

A ground-up suspension of a mesentric gland was injected into a guinea pig, which showed no evidence of infection after six weeks.

MONKEY 15.—On January 29, 1940, the abdomen was opened and the spleen brought out and retained in a subcutaneous pocket in the abdominal wall. This was done in order to observe whether there would be early dissemination of organisms in this organ. On February 17, after the wound had healed, the abdomen was opened by another incision and a nodule was stitched to the omentum. The spleen was aspirated every three days, from February 21 to March 11; examination for acid-fast bacilli gave negative results. On March 16 the spleen was removed for sectioning, no acid-fast organisms were found. After the wound had healed (March 25) potassium iodide was administered, beginning with 1 grain and gradually increasing until 100 grains per day was reached after three months; this dose was maintained for one month. The animal tolerated the drug well. There was no evidence of dissemination of the bacilli.

On August 22, 1940, the animal was given a fourth inoculation. The wound became septic and a few acid-fast organisms were found in the discharge. The wound slowly healed and the acid-fast organisms disappeared. The animal is still under observation.

SERIES C

In this series an attempt was made to block the reticuloendothelial system by injections of India ink. The injections were given in courses which consisted of three injections of usually, 10 cc. of a 5 per cent suspension of the solid ink in distilled water, on three consecutive days, with an interval of 15 days between each course. In monkeys 12 and 14, the ink was first injected intravenously. Monkey 12 received 18 courses, and monkey 14 received 17. Subsequently, owing to blockage of the veins, the ink was given intraperitoneally; 14 and 12 further courses, respectively, were given by that route. Monkey 13 was unable to stand the intravenous treatment, and consequently all injections except the first were made intraperitoneally; in all, 36 courses were given.

MONKEY 12.—This animal was splenectomized and inoculated on March 6, 1939. Reinoculations were done on June 12 and September 23 and on February 21 and August 22, 1940. After each inoculation the wound healed without difficulty. The results to date have been negative.

MONKEY 13.—On March 6, 1930, 20 cc. of the India ink suspension was injected into the vein—the only injection given by that route—and subsequently 35 injections were given intraperitoneally. On March 20 the animal was splenectomized and inoculated. Impression smears from the spleen showed that about 25 per cent of the macrophages had taken up the dye. The operation wound healed completely.

As this animal took the anesthetic very badly and was acutely ill for several days after the operation, it was decided not to repeat the inoculation by the abdominal route. On May 6, therefore, 10 cc. of a heavy sus-

pension of leprosy bacilli was injected intraperitoneally. This animal showed no signs of infection after 1 year and 8 months. It was killed on November 8, 1940, for examination.

Postmortem examination.—General appearance: Much emaciated, the whole skin stained bluish-black. The liver was jet black. The omentum and mesentry, in a similar condition throughout, were matted together and adherent to the viscera and parietal peritoneum. A small wart-like growth was seen adherent to the left side of the anterior parietal peritoneum; whether this was growing from the mesentery or from the peritoneum could not be clearly made out, because of the discoloration and other changes due to the India ink. Apart from partial collapse of the lungs, no other abnormalities were noticed.

Bacteriology: Smears from the wart-like growth showed large numbers of acid-fast organisms in clusters and numerous globi, some of which were intracellular. Smears from the mesenteric and groin glands showed a few acid-fast bacilli in clusters and fairly numerous single ones. Smears from the kidney showed scattered acid-fast bacilli, with a few clusters but no globi. Smears from the liver and bone-marrow were negative. Ear clippings showed a cluster of acid-fast bacilli in one field, and a few acid-fast rods in several others.

Suspensions of kidney and lymph glands injected into a guinea pig and cultured on Dorset's and Lowenstein's media gave negative results.

MONKEY 14.—This animal was splenectomized and inoculated on March 20, 1939, and reinoculated on May 5. After the second operation the wound broke down and discharged acid-fast bacilli but healed completely after a few weeks. On May 6, 1940, a third nodule was implanted in the peritoneum, the wound healing without difficulty. The animal is still under observation.

In view of the fact that the lepromin test has come increasingly into prominence within the last few years, and because it is now generally regarded as indicating the presence or absence of cellular resistance to *M. leprae,* we thought that it might be worth while to apply it to our experimental animals and to normal controls. The antigen was made according to the method of Muir.

In all of eighteen normal, uninoculated monkeys tested, the reaction was consistently negative. The experimental monkeys were then tested, and the results of repeated injections of those discussed in this report are shown in Table 2. (See page 100.) All animals, experimental and controls, were also tested with a similar preparation of rat leproma (Stefansky lepromin); all gave negative results.

It will be seen from the table that there is a tendency for a strongly positive lepromin reaction to develop after the third inoculation. The exception to this statement is seen in series c, in which only one animal (monkey 14) developed a slightly positive reaction. Monkey 12 was occasionally slightly positive, but this reaction quickly gave place to a negative one.

DISCUSSION

The most interesting observations in the continuation of this work were seen in series B, monkeys 6 and 9, in both of which an erythematous rash appeared. In 6 the rash persisted, but in 9 it disappeared for a time, to reappear a few days before death. In both monkeys there was discovered an organism which was partly acid-fast but not consistently of that character. Nonacid-fast saprophytic organisms were grown in culture, among them being numerous diphtheroids. We were not convinced that these organisms had any relationship to the leprosy bacillus, although among them there were acid-fast organisms which closely resembled it. The principal reason for not regarding this organism as *M. leprae* is that there appears to be a similar acid-fast one which can be isolated in small numbers from the eyebrows of normal monkeys. It is believed probable that, under the unnatural diet administered to monkeys 6 and 9, this organism became pathogenic and produced the lesions which have been described.

Whether this organism is the same as the one Collier has described cannot be definitely ascertained, but it is interesting to note that both of these animals were fed on colocasia and that they developed an erythematous rash. This was also a prominent sign in Collier's monkeys. While we ourselves consider this infection to be due to a saprophytic microbe which probably bears no relationship to *M. leprae,* we cannot deny the possibility of its being a stage in the development of the latter. We have failed, except perhaps in monkey 6, to produce progressive lesions. The organisms isolated from these animals were very different morphologically from those demonstrated in our original monkey 1, which we have no doubt was the leprosy bacillus.

The paralysis that developed in both of these monkeys is difficult to explain unless it was a cage paralysis. No bacteria were found in the nerves. The factor in favor of a cage paralysis is that both of them had been under confinement for a longer period than any of the others except monkey 3. The paralysis did not appear to be a vitamin B_1 deficiency, for the diet was adequate in that respect; furthermore, the paralysis in monkey 6 did not respond to large doses of that vitamin.

If, as we believe, the organism which was isolated from these monkeys is not *M. leprae,* it is possibly not identical with the one described by Collier and therefore we cannot confirm or deny his experimental work. The reasons for our failure may be threefold:

(1) *Macacus sinicus (M. radiata)* was used instead of *M. rhesus (M. mulatta).*

(2) The colocasia was originally boiled, and therefore may have lost some of its sapotoxin.

(3) Subsequently colocasia was given raw, but it may possibly not have contained sufficient sapotoxin.

We hardly think the reason for our failure lies in the species of monkey used, for Collier claims also to have infected pigs. The other objections are being met by comparing our colocasia with the variety in Thailand (Siam). This work is continuing and a further report will be issued in due course. In view of the possibility of finding acid-fast bacilli in normal monkeys, any conclusions drawn must be subjected to the most rigorous criticism.

In series C the animals were periodically injected with India ink for the purpose of blocking the reticulo-endothelial system. It was hoped that, thereby, there would be a greater chance of securing dissemination of the leprosy bacillus and therefore of establishing the infection. The postmortem findings in monkey 13 are of decided interest. There was apparently definite evidence of dissemination of that organism, for the acid-fast bacilli discovered were morphologically similar to it and very different in appearance to those obtained from 6 and 9. That is all that can be claimed, however, for there were no clinical signs such as were noted in our first monkey. Whether or not manifestations of clinical leprosy would have developed later cannot be said, but because of the long period since the last inoculation—1 year and 6 months—we regard it as probable that these organisms would ultimately have disappeared. The fact that apparently definite acid-fast bacilli were found in an ear clipping suggests that this should be a routine examination before it is concluded that there has been no dissemination of the inoculated germ and before an animal is killed.

It is somewhat surprising, in view of these failures to produce disseminated lesions that monkey 1, in the first experiments reported, had extensive lesions in which there were found large numbers of acid-fast bacilli with characteristics of *M. leprae*. As previously stated, it may be that this was a chance animal, susceptible to leprosy. Success has not been attained since then, even after six inoculations as in the case of monkey 3; neither have we had any conclusive results in those monkeys which were injected with India ink, in spite of an extensive blocking of the reticulo-endothelial system. Perhaps if monkey 1 had not been killed it would have overcome the infection and the lesions would have disappeared.

All of the monkeys except those in series C tended to develop strongly positive reactions to lepromin after the third inoculation. If, as is generally accepted, a positive reaction indicates resistance

to leprosy, this may explain why the animals did not acquire the infection. It is generally held that the reticulo-endothelial system is intimately connected with the development of resistance, but if this is so there should have been a better chance of the India ink monkeys becoming infected. That does not appear to have been the case, unless monkey 13 can be considered to have been infected. We do not think that happened; we think merely that the bacilli became disseminated without the development of progressive lesions; therefore, no claim of actually producing leprosy can be made. The result in that animal, however, and the fact that it was consistently negative to lepromin, suggests that work might be continued along the line of reticulo-endothelial blockage. The results of these experiments are still inconclusive, and the mechanism of resistance still remains only partially explained.

SUMMARY AND CONCLUSIONS

A further report on inoculation work with splenectomized monkeys is made, and the difficulty in producing disseminated lesions in that animal is stressed. The apparent success of the first experiment, previously reported, has not been repeated. It is suggested that one of the reasons for the failure of development of leprosy in experimental animals is that resistance develops after multiple inoculations, as shown by the strongly positive lepromin reaction that developed in most of our animals which had had more than one inoculation. Some support to this hypothesis is given by the postmortem results in monkey 13, in which there was apparent dissemination of the leprosy bacilli inoculated, but it cannot be claimed that this animal developed leprosy.

In two animals, both of which had been fed on colocasia, an acid-fast diphtheroid was found. In both of them erythematous lesions developed, and both showed signs of paralysis. Reasons for believing that the organism encountered was not *M. leprae* are given. No definite conclusion on this finding can be drawn because it was noted that an acid-fast bacillus, similar in morphology to that obtained from the erythematous lesion, can be demonstrated occasionally in the eyebrow of the normal monkey.

The only definite inference that can be drawn from these experiments is that in monkey 1 of the previous series, and in monkey 13 of this series, there was evidence that the leprosy bacilli had become disseminated. There was, however, no evidence that progressive lesions had developed and therefore none that leprosy was actually produced.

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