The Transmission of Leishmaniosis by means of Cultures, and the Mechanism of the Natural Immunity in Rats and Guinea-pigs.

By

Dr. Arrigo Visentini.

With Plate 21.

Attempts to transmit to animals the infection of Leishmania by means of cultural forms have not always had constant results up to the present.

Nicolle (1909) first inoculated dogs and monkeys (animals notoriously susceptible to leishmaniosis) with 1 c.c. of a culture, but without result; afterwards, however, in collaboration with Manceaux, he obtained positive results by inoculating Macacus cynomolgus and M. sinicus, reproducing the infection with the clinical picture of infantile leishmaniosis.

Novy (1908), by means of cultures obtained from Nicolle, had already succeeded in infecting dogs and afterwards other laboratory animals (?) by inoculating considerable quantities of the parasites, in the case of one dog about 270 cultures in fifty injections. Subsequently, however, Novy (1909) established the fact that a single injection of a suspension obtained from a score of culture-tubes is sufficient to produce leishmaniosis in dogs.

The rabbit has so far been proved to be susceptible only to corneal injection and to the forms occurring in the hæmatopoietic organs. Volpino, by scarification of the cornea with material obtained from a dog infected with experimental leishmaniosis, produced a keratitis similar, up to a certain

point, to that of experimental syphilis. In the circumscribed corneal lesions provoked by Volpino there were found typical Leishmanias, contained in the large mononuclears.

Basile and I have repeatedly inoculated large quantities of cultures in various ways, including the corneal method, without succeeding in our object. On the other hand, a single intra-peritoneal injection of a culture of Leishmania at the eighth transplantation, about fifteen days old and very rich in flagellate forms, was found by Franchini[1] (1911) to be sufficient to produce in a guinea-pig an infection with Leishmania which ran a course that might be termed acute. After six days the animal began to lose flesh and to show febrile symptoms; after twenty-six days the depression previously evident had become very marked. The guinea-pig was sacrificed before spontaneous death intervened. Its weight had diminished by almost half of the original amount. At the autopsy the liver and spleen were found to be enlarged; the marrow of the long bones was very abundant and of a reddish-brown colour; the supra-renal capsules were also enlarged. In the spleen, liver, bone-marrow, peripheral blood, supra-renal capsules and kidney Franchini found forms of Leishmania more or less numerous, but always extra-cellular, a state of things which, in the author's opinion, is in relation with the septicæmic type of the infection.

In the guinea-pig, rat and mouse, Laveran and Pettit (1909) have obtained slight infections of Leishmania localised constantly in the peritoneum as the result of intra-peritoneal, intra-hepatic, and even subcutaneous injections of an emulsion of organs of a dog infected with leishmaniosis. But by means of cultures infection was not obtained in the mouse, even after injections repeated many times and with increased doses. This was established by Delanoë, who has thrown light on the mechanism of the natural immunity possessed by the mouse against the cultures, showing that in

[1] It is well to point out that Franchini, when he uses the name L. donovani, means (at least so I believe) the Leishmania which is the specific agent of kala-azar in Italy.

the peritoneum a very active phagocytosis of the flagellate forms takes place, so that after a short time they are broken up and completely destroyed.

Continuing previous researches left incomplete, I have again undertaken inoculations of guinea-pigs and white rats with cultures, having at my disposition besides the strain obtained by myself from a young patient, Rocca V—, of Bovalino Calabro, other strains also which I owe to the kindness of Mesnil and Jemma. While the cultures of the Pasteur Institute of Paris, originating from Tunis, had been kept alive for some years by successive transplantations, mine and those of Jemma were very recent and still in the first subcultures. The cultures used were at the height of their development, some in the first days of the subculture (3, 5 or 7 days), others older (10, 15 or 20 days) and others very old (1, 2 or 3 months). The object was to inject all the various forms which have been described in the cultures of Leishmania. The quantity injected was almost always 2 c.c., sometimes 4 c.c., for animals of medium size, guinea-pigs weighing about 300 gr. and young rats. In some cases 1 c.c. of the culture was injected, to try the effect of injecting the animals once, twice, thrice or four times. Twenty guinea-pigs and 10 rats were inoculated.

The method of injection was for the most part intra-peritoneal, and only in a few cases was the material of the culture injected under the skin or directly into the current of the circulation, by the vein of the tail of the rats or by means of heart-puncture—a method which seems to me preferable since it does not present excessive difficulties of technique.

Some of the animals died spontaneously, two guinea-pigs and two rats, in the course of the investigations, as happens often with these animals in the laboratory from causes extraneous to the experiments, and of these I made a rigorous examination in search of the Leishmanias; only one rat and one guinea-pig were lost without examination, and of these, of course, I have taken no account. The others were killed

by chloroform at various intervals of time from the first injection, from an hour and a quarter to ninety-three days after, and the autopsy was performed immediately. Both the guinea-pigs and the rats showed no signs of loss of flesh; some of them were even increased in weight. While they were still living, with a glass pipette having one end drawn out into a capillary tube I took from the peritoneal cavity a drop of yellowish liquid, slightly turbid, containing, as we shall see further on, numerous leucocytes, chiefly mononuclears.

The subsequent autopsy revealed nothing noteworthy; the internal organs, spleen, liver, kidneys, bone-marrow and lungs were not increased in volume or changed in appearance or in any way different from those of healthy animals. Of these organs I made smears which I fixed afterwards either in methyl alcohol or with osmic acid vapour and absolute alcohol and stained with Giemsa's stain. The most careful examination has never enabled me to discover a Leishmania, either in its usual form or in the flagellate leptomonad or other type, even when the autopsy followed close upon the inoculation of the cultures into the peritoneum.

Both of the blood and of the internal organs I have tried in each animal numerous cultures in blood-agar (method of Novy and Nicolle), and I have never obtained development of Leishmanias; the culture-tubes remained perfectly sterile, save for some rare exceptions (see below).

I think, therefore, that I am able to establish the point that guinea-pigs and rats possess a natural immunity against the cultural forms of the Leishmania of the Mediterranean basin, alike whether the strains are recently isolated or of long standing and whether the cultures are new or old subcultures.

The case of the infection of the guinea-pig obtained by Franchini with cultures remains, therefore, unique and must at least represent a somewhat rare event. It was a case perhaps of a single animal extraordinarily and exceptionally

receptive. Exceptional at least are the observations which Franchini describes. He found parasites in the peripheral blood and free in the plasma, and similarly all the forms of Leishmania found in the liver, spleen and bone-marrow were extra-cellular. It is, however, usual in Leishmanial infections, by general agreement of all observers, for the parasites to be always or nearly always contained in the protoplasm of the large mononuclears, and it has not yet been shown that they can occur free outside this their natural situation. Unfortunately the drawings which accompany the work of Franchini are so obscure and so little demonstrative that they do not permit one to judge whether one is dealing with Leishmania, the more so since the methods employed by the author are certainly not those the best adapted to obtain elective staining, but are even scarcely sufficient for diagnostic purposes, especially when the parasites, as in this case, are rather scarce.

In order to study rather more closely the mechanism of the natural immunity possessed by guinea-pigs and white rats, and at the same time to avoid any objection to the facts observed by me, I have carried out a double series of investigations, morphological and cultural, with the object of following step by step the fate of the flagellated forms in the organism of the experimental animals.

Having injected into the peritoneum 2 c.c. and sometimes 4 c.c. of a culture very rich in flagellated forms, I drew off every five minutes some peritoneal fluid with a sterile capillary glass tube and examined it fresh with the microscope, making at the same time some preparations for staining. The fact is quickly observed that the parasites undergo absorption rapidly by the leucocytes in the peritoneal cavity.

Prior, however, to describing the phenomenon in greater detail some points of technique may advantageously be dealt with. I have performed the experiment on a sufficiently large number of animals, guinea-pigs and rats, in such a way as to be able to perform, in each animal, only one or two punctures of the peritoneum, with the object of avoiding

those alterations which are produced by the simple fact of operative lesion. With regard to this I can confirm the observation of Delanoë for trypanosomes. When the puncture of the peritoneum is repeated several times, alteration and death of the Leishmanias take place by the probable extrusion of trypanolytic substances from the leucocytes into the plasma, while if the peritoneum be not punctured the flagellates that have remained free are preserved living and with normal structure, and in this condition are engulfed by the leucocytes.

Of the liquid extracted from the peritoneal cavity I have made preparations in the fresh condition and smears on slides which I have fixed while still wet in vapour of osmic acid for five seconds, and afterwards, when dry, in absolute alcohol for a quarter of an hour—a method of technique which also gives very good results for staining the flagellates in the cultures. The smears should be very thin, and it is often useful to follow the staining by very short differentiation in the solution of tannin according to Unna.

I have made examinations of the peritoneal liquid every five minutes after the injection up to two hours and then every half hour up to four hours, repeating the experiment in a fairly numerous series of animals. In the fresh state it is seen in the first few minutes after the injection of the culture that the number of the Leishmanias, truly enormous in 2–4 c.c. of a rich culture, is already to some extent diminished, and not by the fact of the greater dilution alone; very many flagellates are still free and very many are mobile, perhaps even more than in the cultural liquid, but it is not difficult to come upon others adhering or united to leucocytes, either by the posterior end or by the flagellum, preserving, however, in all cases a certain mobility. When they are completely engulfed in the leucocytes they are seen in its protoplasm as rounded bodies, but at this point it is easier to recognise them and to follow their modifications in the stained preparations. In the fresh state I have found in the guinea-pig some Leishmanias free and mobile up to an hour

or an hour and a half after the injection of 2 c.c. of culture into animals of 250 to 300 grammes in weight. In the rat I did not succeed in seeing free forms after an hour and a quarter.

In the stained preparations these various phenomena are very evident, and it is possible to follow step by step the various phases of alteration to which the Leishmanias are subjected.

One fact which deserves special emphasis is that the parasites are found always in the large mononuclears and in them alone, that is to say in just those elements which contain them in kala-azar and oriental sore. In the rare cases in which a true polynuclear is found apparently containing a Leishmania, the possibility cannot be excluded that it is simply superposed rather than engulfed. In the protoplasm of the mononuclears they become more rounded in form, preserving sometimes all their constituent parts and traces of the flagellum. Thus it is possible in some parts of the preparations to have the impression that it is a case of simple modification of the form of the Leishmanias and that they have found in the mononuclears a habitat adapted to them; but for the most part they present obvious alterations. The protoplasm becomes clear or turbid, the contours of the parasite disappear, the flagellum becomes thickened and breaks up; sometimes the trophonucleus or the kinetonucleus is wanting, or these bodies lose their sharp contours and their special characteristics and are reduced to shapeless masses of chromatin.

After fifteen to twenty minutes very many mononuclears are observed containing Leishmanias profoundly altered, represented after twenty to thirty minutes by remains of nuclei near which a portion of the flagellum is to be noted. The profound modifications of the phagocyted parasites continue, and after two to three hours there are found only chromatinic granules with masses of protoplasm stained intensely in reddish violet, representing the last vestiges of the destroyed Leishmanias.

Even after thirty minutes up to an hour the first stages of the phagocytosis are still to be observed; mononuclears which contain forms greatly altered show others in process of being engulfed, while other flagellates still quite intact remain free and perfectly mobile in the serum, like that shown in Fig. 7, twenty-five minutes after the injection.

I have also made, as already stated, preparations of the peritoneal exudation of the animals sacrificed at various periods up to ninety-three days after the injection of the cultures and I have never seen Leishmanias or remains of them in the mononuclears, although I have directed special attention to this point, because Laveran and Mesnil have observed the peritoneal infection up to fifty-nine days after the injection of emulsion of organs of a dog infected with experimental leishmaniosis and have obtained subcultures of this fluid.

Contemporaneously with the examination of the peritoneal fluid I have repeatedly made an examination of the peripheral blood, and, in animals sacrificed, I have made smears of the various organs; in spite of the most patient investigation I have never met with any form of Leishmania or any flagellate parasite in my numerous preparations.

The facts described permit me to assert that we are dealing with a process of phagocytosis pure and simple; it cannot be admitted that the flagellate leptomonad forms are transformed into Leishmania-forms and as such continue to live in the mononuclears, producing a slight infection localised in the peritoneum, of the type of that obtained by Laveran and Pettit, because we are witnesses here of a gradual alteration and destruction of the parasites by a process which leads to the final disruption of all remains of the injected Protozoa. It is scarcely necessary to add that in the forms engulfed every sign is lacking of a process of multiplication. Moreover the results of attempts to make cultures confirm fully this interpretation, and give a proof of it which I think can be regarded as absolute. In every animal killed with chloroform I have made cultures in blood-

agar according to Novy-Nicolle of blood taken aseptically from the heart, spleen, liver, bone-marrow, and in some cases of the peritoneal fluid. The results were constantly negative except with the peritoneal fluid a short time after the injection of the culture, when it is still possible to find by microscopic examination some Leishmanias living and mobile. Thus after an hour and a quarter in one rat I was able to obtain a new culture from the peritoneum, while after two hours the result has always been negative.

In complete accord with the result of the histological examination I have never obtained development of flagellates in a culture from the blood or from the internal organs, not even by injecting into the peritoneal cavity or directly into the circulation 4 c.c. of cultural fluid very rich in Leishmanias. These flagellate Protozoa would not, therefore, pass beyond the peritoneal barrier in the guinea-pig and the rat.

I have stated above that I have injected the cultures of Leishmania directly into the blood in some animals. Although my investigations may not be sufficiently numerous, being limited to one guinea-pig and one rat alone, I have noted in the blood immediately after the injection a marked leucocytosis with prevalence of mononuclears, which reached its maximum degree after five to ten minutes, was maintained for half an hour, and then diminished gradually. In these cases also I was never able, even soon after the injection, to find flagellate Leishmanias in the blood, nor have I seen phagocyted forms. The animal has not contracted any infection.

Summary.

Guinea-pigs and rats possess natural immunity to a high degree against the Leishmania of the Mediterranean basin in the flagellate stage in cultures on blood-agar (method of Novy-McNeal-Nicolle).

This immunity is exclusively of phagocytic nature. The Protozoa injected into the peritoneum become rapidly en-

gulfed by the leucocytes, the mononuclears exclusively, and undergo gradual and progressive alterations, ending in their complete destruction, so that by the end of an hour or an hour and a half after the injection of 2 c.c. of a culture in full development, free flagellates are no longer to be found in the serum, and after two or three hours even the last vestiges of them are almost destroyed in the protoplasm of the phagocytes.

The Protozoa do not pass beyond the barrier of the peritoneum and do not find their way in this manner into the blood or the internal organs.

These data of observation receive full confirmation in the results of the cultures. It is possible, in fact, to obtain subcultures from the peritoneal fluid up to about one hour after the injection of 2 c.c. of liquid of condensation very rich in Leishmanias, but not beyond this period, nor are subcultures obtained from the blood or organs of the animals in experiment.

I desire to express my gratitude to Dr. Martin for having received me with so much kindness and hospitality at the Lister Institute of Preventive Medicine, London, of which he is the Director, and for having provided me liberally with the means of research; I also thank Dr. Bayon warmly for assisting me in every way during my stay in this Institute.

Lister Institute of Preventive Medicine,
London,
May 25th, 1912.

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DESCRIPTION OF PLATE 21

(From smears of peritoneal fluid),

Illustrating Dr. Arrigo Visentini's paper on "The Transmission of Leishmaniosis by means of Cultures, and the Mechanism of the Natural Immunity in Rats and Guinea-pigs."

[The figures are drawn with the camera lucida at a magnification of 2000 diameters.]

Fig. 1.—Five minutes after the injection of a culture of Leishmania, showing one parasite absorbed, and another in process of absorption, by a mononuclear leucocyte.

Fig. 2.—Ten minutes after injection; a leptomonad form absorbed, another held fast by its flagellum.

Fig. 3.—Thirty minutes after injection; a mononuclear containing three parasites, in process of destruction, and in the act of absorbing a fourth.

Figs. 4 and 5.—Fifteen minutes after injection; mononuclears containing Leishmanias in process of destruction.

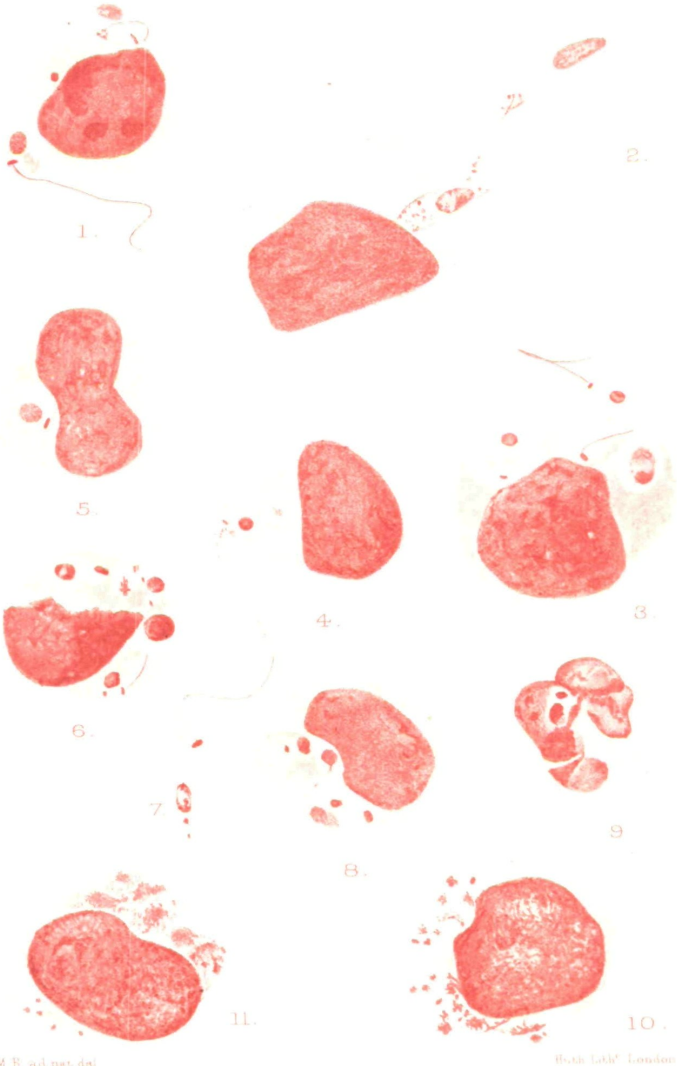
Fig. 6.—Twenty minutes after injection; mononuclear containing the remains of several parasites.

Fig. 7.—Free Leptomonas-form twenty-five minutes after injection.

Figs. 8 and 9.—One hour and a quarter after injection; a mononuclear containing remains of several parasites and a polymorphonuclear apparently containing (?) a parasite.

Fig. 10.—Two hours after injection; mononuclear containing the last vestiges of several parasites.

Fig. 11.—Three hours after injection; as the last.



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PHAGOCYTOSIS OF LEISHMANIA