# ROSETTES IN RAT LEPROSY*

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IT IS commonly stated that in the acid fast bacterial disease of rats called "rat leprosy," there are no globi[1, 2] and, further, that this is one of the distinguishing features between it and human leprosy. There is, however, no agreement as to the nature of the globi in human leprosy[3] except that they are roughly spherical masses of bacilli embedded in a substance known as *Schleim* and enclosed in a sort of capsule.

The purpose of this paper is to report the occurrence of certain clumps of bacilli in rats, which resemble the human globi in some ways and differ from them in others, and which we designate "rosettes" because the bacilli in them exhibit a marked radial arrangement.

## OBSERVATIONS

Photomicrographs of rosettes, seen in sections of nodules stained with the Ziehl-Neelsen fuchsin method for acid fast bacteria and counterstained with Harris' hematoxylin for general structural details, are presented in the illustrations, for which we have to thank Dr. James O'Leary.

The rosettes are usually more definitely spherical in shape than the globi. In fixed and stained preparations their size varies from a diameter of 7 to 35 microns. The maximum is less than that of the globi, which attain a diameter of about 90 microns in our human specimens.

Our strain of rat acid fast bacilli was obtained through the kindness of Dr. E. L. Walker. In our experiments inoculations were made subcutaneously into the anterior abdominal wall of 121 rats. Bacilli-laden cells were fairly numerous at the site of inoculation 11 days later. Animals were killed at intervals, and rosettes were observed 120 days after inoculation. No determination was made as to the time of their first appearance.

It is possible to characterize fairly definitely the cells in which the rosettes develop. They are usually larger than most of the cells of the nodule and occur chiefly in the periphery of the nodule near the blood supply or near vascularized septa of connective tissue penetrating into

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the nodule. As a rule, each cell has three to six, or even more, nuclei of rather uniform size located in the peripheral cytoplasm, generally to one side. The cells resemble those said to be produced by fusion of monocytes. They are not found in the degenerating centers of nodules or near the surface of open ulcers. We have never seen signs of mitosis or of amitosis in them.

The first indication of the development of a rosette is the clumping together of a few bacilli at some point in the cytoplasm of the cell. Their orientation, to begin with, is not very definite. Sometimes a bacillus is bent in a U-shaped fashion, so that both ends stick out into the cytoplasm, while another, in a much shallower U, extends almost from one side of the clump to the other, and a few shorter ones more definitely stretch from the inside of the clump outward. As the clumps become denser, this radial arrangement is accentuated and the cytoplasm at the periphery of the rosette begins to look rather empty. Figure 1 shows this stage in a comparatively small cell of a subcutaneous nodule. Many bacilli are superimposed in the photomicrograph; but, in the original preparation, their individual outlines can be distinguished.

The clear halo about such a rosette is better represented in figure 2. It shows a larger cell, with more bacilli-charged cytoplasm. The outer limits of the halo are more irregular than those of globi of this size in human leprosy.

Outward radiation of the bacilli, forming the rosette, is best demonstrated in figure 3, from part of a liver nodule, and is superficially suggestive of a ray-fungus.

The rosette is more compact and the halo wider in figure 4 which, together with the following illustrations, is taken from subcutaneous nodules. The halo is limited on the upper right and middle left sides by the cell membrane, which appears to be distended as if by fluid under pressure. The cytoplasm on the upper left and lower right, which is nucleated, contains but few bacilli, though perhaps a section taken through this cell at a different level would have shown more.

Figure 5 illustrates a large cell with a well formed rosette of closely packed organisms. Bacilli are also quite numerous in the cytoplasm outside the halo.

The formation illustrated in figure 6 is unusual. The radiating bacilli are clumped in leaf-like masses with clear spaces between them, and the halo is narrow. There are many bacteria in the cytoplasm of this cell outside the rosette.

In figure 7 a rosette of about the maximum size is shown. The limiting

cell membrane is distinct in the original preparation, but only parts of it can be seen in the photomicrograph. The rosette looks double by reason of the leaves on the right and left, which present a sort of frayed appearance. It is surrounded by a wide halo. The cytoplasm of the host cell is reduced in amount, but the spherical outline of the cell membrane again suggests fluid under pressure.

Figure 8 represents an unusual rosette. The bacilli form a dense palisade about a central clear area. This is, however, an extreme case. Many intermediate stages between its formation and that of the rosette depicted in figure 3 have been observed, but cost of illustration prohibits presentation. In figure 3 the center of the rosette is noticeably clearer than the mass made up of radiating bacilli. Often the clear area is larger and the bacilli appear to radiate from two centers like the mounts of two fans, each extended to form a semicircle only. In our preparations, the clear central area does not enlarge beyond the stage indicated in figure 8.

Much larger giant cells, like the one represented in two consecutive sections in figures 9 and 10, are but rarely encountered in dense nodules. They are more frequently met with in adjacent small masses of subcutaneous lymphoid tissue. The bacilli are numerous in a semilunar band placed between the nuclei of the giant cell and the large area of cytoplasm free from nuclei. This is best seen in figure 10. On the left are the nuclei, next is the band of bacilli, and on the right is the relatively clear cytoplasm. We interpret this to mean that in such cells physico-chemical conditions favorable to the multiplication of bacilli are sharply limited to a certain portion of the cytoplasm, in which region the rosettes develop. Three distinct and several indistinct ones can be distinguished in figure 9, while in figure 10 a rosette, seen in the lower right portion of figure 9, has been cut more nearly through its largest diameter.

By means of serial sections we eliminated the possibility of interpreting tangentially cut rosettes as young ones, in which usually there are no clear central spaces.

Since the nodules grow by the addition of bacilli-charged cells at the periphery, it follows that in the central parts, which often degenerate, the presence of bacilli in the cells is of longer standing. The fact that we do not see the rosettes in the centers of the nodules indicates to us that they disappear before the cells containing them are forced to occupy a more central position by the accumulation of more and more cells on the outside. It was difficult to find the various stages that cor-

roborate this supposition. It can be said, however, that towards the center of the nodules the rosette in a given cell occupies more of the cytoplasm, as is demonstrated particularly by figure 7. The cell is thus handicapped, and the frayed rosette contained in it possibly indicates the beginning of disintegration. After the rosette is broken up, it becomes unidentifiable.

Though detailed study will undoubtedly yield a more accurate sequence of stages in the development of rosettes, it is significant that all those mentioned above may be found in a single section, even though only a minority of the cells contain rosettes. Evidently the stimulus to rosette formation, whatever it may be, does not sweep through the lesion but becomes effective locally in individual cells at different times. Over a period of at least two hundred days, in our series, rosettes were being formed in a few of the large multinucleated cells described.

Wolbach and Farber,[4] on the other hand, in studying the nuclear inclusions in the salivary glands and other organs of infants, were perplexed by their uniformity in size and appearance, there being no small forms that might be interpreted as stages in formation. Cowdry and Scott[5] presented evidence in their study of intranuclear inclusions in the salivary glands of the monkey, *Cebus fatuellus*, that this uniformity is occasioned by the fact that the inclusions were terminal stages in dead cells,[6] and for this reason a progressive series from small inclusions to large ones, indicative of an active process, as occurs with these rosettes, was not to be expected.

We have tried many techniques in an attempt to discover what structure, if any, is present in the center of developing rosettes. The bacilli radiate like some materials do about a centrosome, but in much more dense formation. However, no centrosome has been found. It is interesting to note that the microincineration method of Scott[7] does not reveal any concentration of minerals in the rosettes. In a rat given four injections of trypan blue, very little dye was found in the nodule and none in the cells possessed of rosettes. No progress was made in the study of rosettes in still living cells teased out in isotonic salt solution, because of difficulty in finding them. For this reason we cannot state how much the morphology of the rosettes is distorted by technique. But we did not find a difference depending upon the nature of the fixative—formalin, alcohol, or Regaud's fluid—so that they may not be very susceptible to modification. The irregular halo does not look like a shrinkage space. It may be either emphasized, minimized or but slightly changed by fixation. The method of decolorization for acid fast

organisms leaves very little stain in the cytoplasm, because the hematoxylin used for general details is primarily a nuclear dye. When, instead of hematoxylin, Mallory's triple connective tissue stain is employed, the cytoplasmic background is colored a mixture of blue and orange and, in some cases, each bacillus seems to be in a very narrow uncolored space which calls to mind the relatively large uncolored space that constitutes the halo about the rosette. We are not prepared to commit ourselves as to whether the space, or the halo, or both are produced as an interaction between the living cell and living or dead bacilli.

The bacilli which make up the rosettes are generally of the same size, shape and internal structure as other bacilli in the same cell and in neighboring cells. Usually they are rather long rods and filaments. Within the filaments, granules are often to be observed, especially in thin sections which, on that account, are subjected to more energetic decolorization than the thicker ones. These granules are of approximately the same diameter as the filaments, and only seldom are they larger. The fact that the bacilli look as if they had been alive at the time of fixation of the tissue, means very little, for so also did the dead bacilli which Lowe[8] injected into rats and which caused monocytic reaction and giant cell formation in much the same manner as living organisms do. Denny[3] confesses his inability to tell "which of the acid fast bacilli seen in material freshly obtained from leprous tissues are living and which, if any, are dead but resisting disintegration."

There is no morphological or chemical test for vitality of individual bacilli.[9] But it is interesting to note that in these rats the only place, with the exception of the rosettes, where the bacilli are packed closely side by side, is in the degenerating centers of the nodules. Here the cytoplasm of uninucleated cells loaded with bacilli is reduced in amount as the cells decrease in size without, however, any noticeable parallel alteration in the nucleus. This is often accompanied by a close approximation of rod-like bacilli in masses reminiscent of the "cigar packs" of bacilli in human leprosy. But these bacilli, in contrast to those in the rosettes, are applied to each other through their entire length, and halo formation about them is slight or absent. It is unlikely that in this central part of the nodule close packing together of the bacilli is evidence of multiplication.

The enormous number of bacilli in the rosettes does suggest multiplication, but again, such evidence of multiplication is not worth much when seen in fixed tissue. In very thin sections we could not satisfy ourselves as to the presence of any significant number of longitudinally

splitting organisms. It was difficult to distinguish between overlapping and branching, yet it is by division in this plane that one would expect a figure of this peculiar type to form, since the bacilli stretch out in all directions from a center near which their central ends must be wedged together very tightly. If, on the contrary, multiplication should be by transverse division, which is the accepted method for mycobacteria, and which we certainly do not deny, we would look for a shedding of granules and short rods from the periphery of the rosette, of which process we can find no trace. The alternative, that small organisms formed in this way are drawn inward toward the center by some unknown force, is not plausible.

It is also conceivable that little, if any, multiplication occurs in the rosettes and that they represent a peculiar kind of clumping of living or dead bacilli produced either by bacillary multiplication in other parts of the cytoplasm, or by continued intake of bacilli from the tissue fluid surrounding the cell. Of the two, the latter consideration has least to commend it, since there are not many extracellular organisms.

To reason by analogy is risky, but the tubercle bacillus in respect to acid fastness and some other properties resembles this organism of rats. Obvious differences are for the moment ignored. According to Kahn and Nonidez[10] the formation of granules is a stage in its developmental cycle. They look upon it as "a type of segmentation rather than direct division in which the rate of segmentation surpasses the ability of the elements thus formed to elongate." The loss of acid fastness in the granule and small rod may, in their opinion, be a deprivation phenomenon caused by "insufficient time for the organisms to metabolize the substances essential for their acid fastness." We have no measure of the rate of multiplication of the acid fast bacteria of the rosettes as compared with these tubercle bacilli which lose their acid fastness. Extracellular multiplication is much commoner in human tuberculosis than in rat leprosy. This is another feature in which conditions are not strictly comparable. In the globi of human leprosy, loss of acid fastness is not unusual. In the rosettes, on the other hand, the bacilli are as strongly acid fast as others in the vicinity. Indeed, in our specimens, it is difficult to detect any signs at all of loss of acid fastness.

Perhaps, multiplication in the rosettes is so leisurely that there is plenty of time to metabolize the necessary substances. We mention this merely as one of many possibilities in a very complex problem, not as a conclusion. On the whole, however, it seems to us that the balance of probability is in favor of the tentative conclusion that the rosettes are foci of multiplication of bacilli.

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It has been mentioned that the rosettes do not attain the size of the giant globi in leprosy. There is no noticeable material associated with the rosettes like the *Schleim* in the globi. Yet the rosettes resemble the globi in several particulars: Neither the rosettes nor the globi are early stages in the disease reaction; globi are absent in some types of human leprosy and we cannot yet say that rosettes are present in all cases of rat leprosy; in the formation of both, localized multiplication of bacilli probably takes place, but in the globi a comparable radial arrangement of bacilli is never encountered; in both there is an accumulation of fluid which explains the halo and the signs of distention of the affected cells, which become rounded and swollen, but it is questionable whether the surrounding membrane is of the same nature. In the case of small rosettes (fig. 2) and even of large ones (fig. 6) there is no sharp line of demarcation between the substance of the rosette and the cytoplasm of the cell. Only when the cytoplasm is reduced (figs. 4 and 7) does the cell membrane limit the rosette. With the globi, on the other hand, the membrane is still of undetermined origin.[3] We are investigating its properties and believe it unwise to assume, as most observers do, that it is in all cases of a single nature and origin, because in leprosy more types of cells and systems are directly involved than in this acid fast bacterial disease of rats.

Both diseases are representatives of a large group designated by Long, "Mycobacteriosis." Since there is no reason to suppose that our strain of the rat disease differs from that investigated by others, it follows that rosette formation may be a fundamental characteristic which has been overlooked until now. This could easily happen when chief reliance is placed on hematoxylin and eosin stains, and when coloration for acid fast organisms is resorted to only occasionally. Moreover, the rosettes may not be so apparent after intraperitoneal inoculations of bacilli, although we found a few after careful search, in the liver of the rat given us by Dr. Earl B. McKinley. Whether rosette formation is an occasional occurrence in the spontaneous acid fast bacterial disease discovered by Krakower and Gonzalez[12] in mice and in Johnes disease of cattle as well as in some forms of tuberculosis, further investigation alone will show.

SUMMARY

In rats which have been injected subcutaneously with acid fast bacilli, radiating accumulations of bacteria called "rosettes" appear in certain multinucleated cells at the periphery of the resultant nodules about the 120th day, and become quite conspicuous later on. These rosettes are probably foci of multiplication of the organisms. They differ from the

giant globi of leprosy mainly in their smaller size, constant acid fastness of bacilli, absence of *Schleim* and by their definite intracellular location.

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## DESCRIPTION OF ILLUSTRATIONS

All the illustrations are photomicrographs of 4 micron sections stained with Ziehl-Neelsen for acid fast bacteria and countersigned with Harris' hematoxylin for structural details. The figures are all of sections from subcutaneous nodules in the anterior abdominal wall except figure 3, which is of a nodule in the liver of a rat, sent to us by the courtesy of Dr. Earl B. McKinley. Figures 1 and 2 are taken 193 days after inoculation, and figures 4 to 9, 376 days after inoculation.

Magnification, 1200 diameters.

## PLATE I

Fig. 1. A small rosette is shown within a macrophage, the outlines of which are not seen. Note radial arrangement of bacilli.

Fig. 2. A small rosette within a much larger cell, the cytoplasm of which is crowded with bacilli. It is surrounded by a clear halo of cytoplasm devoid of bacilli.

Fig. 3. A larger rosette, better illustrating the radial arrangement of bacilli. It is surrounded by an unusually dense accumulation of monocytes and macrophages.

Fig. 4. The bacilli which form the rosette are more densely packed together than in figure 2, the clear halo is larger, and its limits are faintly marked. The two nuclei below and to the right belong to the multinucleated cell containing the rosette. The rosette leaves but little cytoplasm unoccupied.

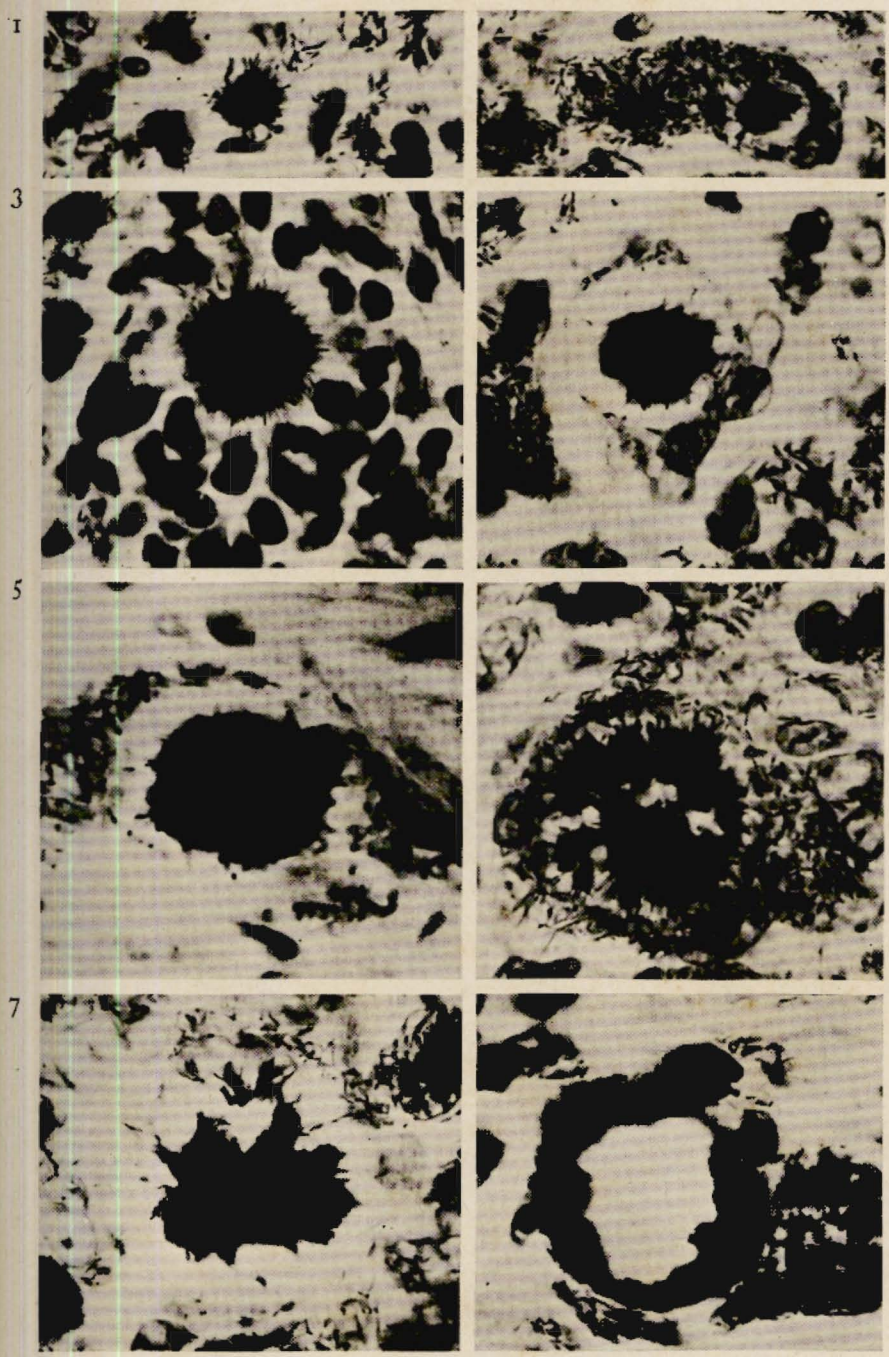
Fig. 5. The rosette is larger, and the other bacilli in the cell are held at a distance from it by a clear halo.

Fig. 6. A rosette with radiating bands of bacilli somewhat separated.

Fig. 7. A large rosette within a clear space, the extent of which may be estimated, though it is not well demonstrated in the photomicrograph.

Fig. 8. In this rosette the bacilli form a dense palisade about a central clear area.

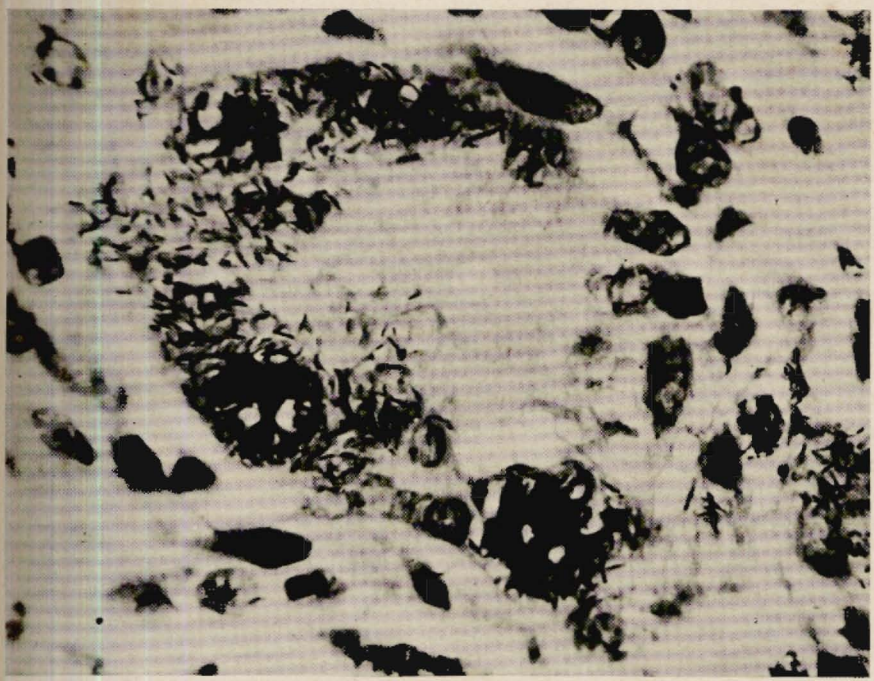
PLATE I



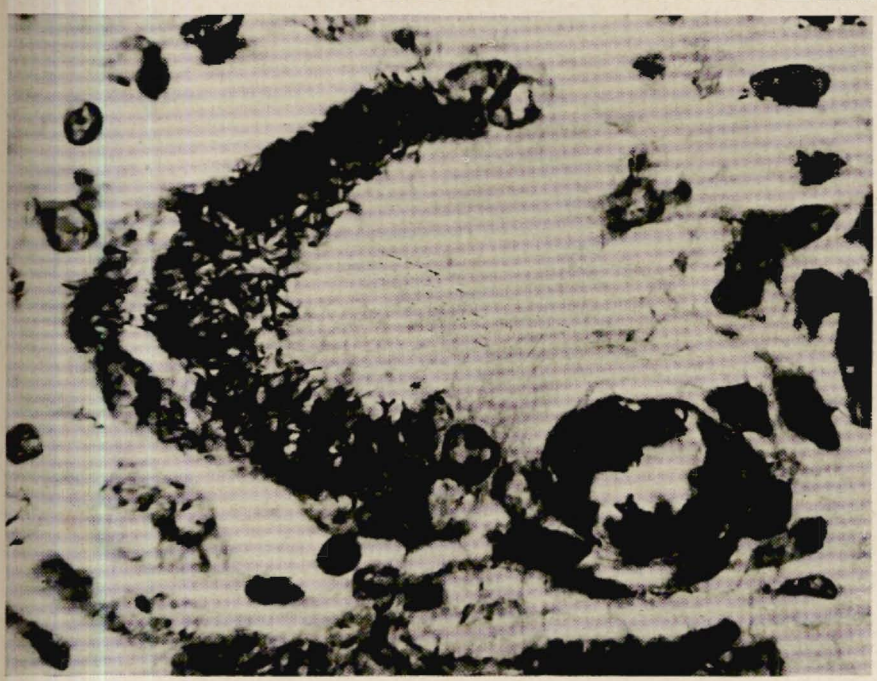
## PLATE II

Figs. 9 and 10. These are taken from neighboring sections of a single large giant cell. Most of the nuclei of the cell are to the left. Between them and the central cytoplasm the bacilli are heaped in a semilunar mass. In figure 9 we see three distinct rosettes in this mass, and in figure 10 we see a deeper section of the rosette shown in figure 9, below and to the left.

PLATE II



9



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