TYPHUS FEVER

II. CYTOLOGICAL STUDIES OF THE SCROTAL SAC EXUDATE IN TYPHUS-INFECTED GUINEA PIGS

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PLATES 22 AND 23

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INTRODUCTION

The scrotal reaction in guinea pigs reacting to Mexican typhus was first described by Neill (1) in 1917. Mooser (2) in 1918 made the important observation that the scrotal sac exudate in animals showing this reaction contained cells greatly distended with minute diplobacilli. These organisms are entirely similar to those seen in the epithelial cells lining the gut of the typhus-infected louse and to those seen in the endothelial cells of specific typhus lesions in man and laboratory animals, (Wolbach, Todd and Palfrey (3)). The etiological relationship of this organism (*Rickettsia prowazeki*) to typhus must be regarded as established.

Maxcy (4) also found *Rickettsiae*-laden cells in the scrotal sac exudate in guinea pigs infected with endemic typhus in southeastern United States. The author (5) found an entirely similar picture in the Wolbach strain of European typhus, but the inflammation of the scrotal sac was usually milder in this strain and occurred only periodically. Previous to these observations, *Rickettsiae* could be demonstrated in mammalian tissues only in small numbers, and the reaction to the organism had always been described as a proliferative response on the part of the vascular endothelium.

Cytological study of the scrotal sac exudate has brought out several interesting points which it is the object of this paper to record. These observations have been made on the Maxcy strain of typhus originating in North Carolina, instead of on the European strain because one

can more easily and constantly get material containing numerous infected cells from this strain; but unquestionably the observations could, with patience, have been made on the Wolbach European strain by taking advantage of the periodically intensified scrotal reaction. The main objects of this study were (1) to determine the origin and nature of the various types of cell present in the exudate and (2) to determine to what extent the various types of cell take up *Rickettsiae* and to what extent the organism multiplies in each type of cell. Incidentally it was hoped that such studies might help to solve the question of whether or not the peritoneal lining cells give origin to any important number of "peritoneal phagocytes."

OBSERVATIONS

In the gross the exudate is not remarkable in appearance. In the early stages it is a glairy greyish white material, in which small white flakes (up to 1 mm. in greatest dimension) are frequently found. Later (after 24 to 48 hours) the exudate becomes firmer and drier and loosely glues together the visceral and parietal layers of tunica. At this stage it is easily peeled off in large thin sheets. By grasping it with the forceps at any point it is often possible to denude the entire surface of the testicle and cremasteric muscle. When sheets of this exudate are floated in Ringer's fluid they have a distinctly membranous appearance and when manipulated with dissecting needles, planes of cleavage appear which are always parallel to the flat surface. It is thus possible to separate the material into thin sheets only two or three cells deep.

Later the exudate either becomes partially replaced by permanent connective tissue or disappears by resolution, allowing a restoration of the serosa to normal. The latter process is the rule in the Wolbach strain of European typhus where the inflammation is relatively mild, and the exception in the American strains.

Microscopic study of the exudate shows inflammatory cells of all types and relatively little fibrin. In the early stages considerable serum is present. Table I shows roughly the composition of the exudate at various stages. The figures in this table were obtained in each case by counting 1000 cells in Giemsa-stained smear preparations. The term "large mononuclear cells" is used in a descriptive sense only

and includes macrophages of whatever origin, desquamated serosal cells and perhaps a small number of young fibroblasts and true vascular endothelial cells. The serosal cells can often be identified in Giemsa-stained smears with a fair degree of certainty. In the first place they tend to occur in small clusters accurately fitted together. Secondly, the serosal cells have rather pale nuclei and in well fixed smears several definite blue staining nucleoli are almost invariably visible, while the nucleoli of the macrophages are not seen.

In supravital preparations more accurate identification of these cells is possible. Of the macrophages about 25 per cent take up dye in the manner characteristic of the monocyte, while the remainder are

TABLE I

	Polymor- phonuclears	Eosino- philes	Lympho- cytes and plasma cells	Large mononuclear cells*
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
First few hrs.	16	1.5	2.5	80
24 hrs. (approximately)	56	4.2	2.1	38
48 " "	41	3.2	6	50
4 days.	10	1.2	4	85
6 " "	1.2	0.2	7.5	91

* Term used in a purely descriptive sense.

probably largely of local origin (histiocytes, clasmatoocytes). The dye-storing cells constitute about 35 per cent of the "large mononuclear cells" of the exudate. The remaining 65 per cent do not store dye and are probably largely serosal cells. It is impossible to recognize young fibroblasts and true endothelial cells (originating in capillary sprouts) among these non-phagocytic cells but these types of cell must be present in very small numbers if at all in the early stages of the process, when the exudate is not adherent.

In smears made during the first 48 to 72 hours, *Rickettsiae*-filled cells are practically always found, and during the first few hours of the reaction such cells are frequently present in every low power field.

Heavily infected cells are best located under low power magnification and stand out as swollen cells with dark purple cytoplasm. Occasionally the cytoplasm is so

packed with organisms that the nucleus is entirely obscured and the cells appear at first glance like masses of homogeneous darkly stained foreign material. In some of these heavily infected cells it is impossible to resolve the individual organisms except along the edge.

In other cells the nucleus appears small and dark, obviously compressed by the growth of organisms in the cytoplasm. With the oil immersion, many cells are also found which contain smaller masses of organisms. Some show one-half or one-third of the cytoplasm packed with organisms, while the uninfected portion of the cytoplasm remains clear. Even when only 15 to 20 organisms are present in a single cell, they are almost invariably in the form of a closely packed cluster, suggesting that the motility of the cytoplasm is not sufficient to disseminate them.

Unfortunately it was not possible to recognize the *Rickettsiae*-filled cells in supravital preparations. By the use of graphite ink, however, it was possible to obtain smears in which the *Rickettsiae* were plainly visible in the cytoplasm of the cells and in which the phagocytic cells could be easily recognized.

The two methods used were (1) to mix the exudate with the ink particles suspended in saline and make smears after 15 to 45 minutes, and (2) to inject the ink suspension intraperitoneally at the onset of the scrotal reaction. The latter method resulted in more satisfactory preparations. 3 drops of ink in 5 cc. of saline were injected. 24 hours after injection 50 to 80 per cent of the polymorphonuclears and 65 to 90 per cent of the macrophages (as determined by supravital preparations) were found to contain definite ink particles, and the greater number of these cells were well filled. This was used as a criterion for the success of the contact between the cells of the exudate and the ink particles.

In smears of this type, 400 *Rickettsiae*-infected cells were counted, 108 of these being completely filled with *Rickettsiae*, 134 partially filled and 158 containing from 6 to 100 individual organisms. Of these 400 infected cells, counted in smears representing four different experiments, only two contained particles of graphite and in each of these two instances it was obvious that the cells had been invaded by (or possibly were simply in contact with) polymorphonuclears and that the graphite particles were originally in the cytoplasm of the polymorphonuclears and not in the *Rickettsiae*-infected cells.

On the other hand, 2000 mononuclear cells containing ink particles were carefully inspected and no *Rickettsiae* were found in any of them. Later several hours were spent in examining these phagocytic cells without enumerating them, and no *Rickettsiae* were ever found in cells which had ingested ink particles.

No organisms were seen in the polymorphonuclears but in other smears as many as ten organisms have been counted in the cytoplasm of some of these cells. This observation had been made only very rarely, however. When the organisms are seen in polymorphonuclears, they are scattered uniformly through the cytoplasm,

as though they had been picked up one at a time. It is probable that they are rapidly destroyed.

These experiments showed in a clear-cut manner that the cells in the scrotal sac which had become infected with *Rickettsiae* and which we are forced to regard as entirely of serosal origin (see observation made on sections below) do not take up graphite ink particles and do not stain supravitaly and that the monocytes and clasmotocytes (histiocytes) which take up graphite ink, do not take up or become invaded by *Rickettsiae* in demonstrable numbers (see Fig. 1). This latter observation agrees quite well with our repeated failure to find organisms within the circulating blood monocytes, and we believe that *Rickettsiae* gain entrance to the blood stream largely in a naked condition by way of lymphatics.

Sections through the scrotum, scrotal sac exudate and testes, stained in such a way as to bring out the *Rickettsiae* clearly, have furnished valuable material for study in connection with the above experiments.

The infected serosal cells are quite loosely attached and great care is necessary to get preparations in which they are intact. The following method has been most satisfactory. The entire scrotum, testes and rectum were removed *en bloc* and placed in Regaud's fluid (20 cc. of full strength formaldehyde solution, 1 gm. of sodium sulfate and 100 cc. of 2.5 per cent potassium dichromate solution). Equally good results were obtained with neutralized and unneutralized formaldehyde. After a few hours the scrotal sacs were gently opened from above downward to allow better access of the fixative to the visceral and parietal tunica. After 48 hours fixation, followed by gentle washing for 24 hours, suitable blocks were cut with a sharp razor, taking care not to disturb the surface exudate. The blocks were dehydrated, cleared in cedar oil and embedded in paraffin in the usual way. Sections were cut as thin as possible and stained overnight in Giemsa solution. Exposure to sunlight is the most satisfactory method of differentiation, since colophonium decolorizes many of the organisms.

In sections made in this way, the topography of the inflammatory reaction may be studied, and more information can be obtained than in a study of smears. The intracellular *Rickettsiae* stain purple and are shown fully as well as in the best smear preparations. They are stained as deeply and sharply as ordinary bacteria. No intermediate stages between definite *Rickettsiae* and granules of indefinite nature are seen, and it seems safe to assume that practically all of the *Rickettsiae* present in any given section are clearly visible.

The outstanding features of such preparations (Fig. 2) are the presence of enormous numbers of *Rickettsiae* in the cytoplasm of the serosal cells, and the extreme difficulty of finding the organism in the underlying tissue or in the cells of the overlying exudate. Organisms are never seen in an extracellular position. In many preparations, more than half of the serosal cells are completely or partially filled with *Rickettsiae*, and as many as twenty-five adjacent cells have been seen, all of which were heavily infected. Occasionally a desquamated *Rickettsiae*-containing cell is seen in the exudate near the serosa, but the cells which lie further out in the exudate (polymorphonuclears, eosinophiles, lymphocytes, macrophages, desquamated serosal cells and connective tissue cells, if any) appear uniformly *Rickettsia*-free.

There is often marked proliferation of the serosal cells, sometimes resulting in concentric whorls and sometimes simply forming a thick layer of heaped up cells, 15 to 30 cells deep. These heaped up cells tend to elongate and resemble fibroblasts, but as has been said, *Rickettsiae* are present almost exclusively in those cells which are in contact with the subserous collagen layer, or, in other words, these cells which occupy the position of the original peritoneal lining cells. Whether this is because the proliferated serosal cells have acquired immunity from their infected antecedents, or because physical or chemical changes associated with their altered position make them unsuitable for the growth of the organisms, would seem to be an important question which we have not been able to answer. Frequently when the original or "basal" layer of serosal cells has become obscured by the reaction, it can be located under low power as a layer of cells filled with purple *Rickettsiae*, just outside of the dense subserosal collagen. Figs. 3 and 4 show a localized area of serosal cell proliferation with *Rickettsiae* present only in the "basal" layer.

The proliferated serosal cells at this stage are embedded in a fibrin clot. Lateral, typical collagen appears around these cells and the transition between fibrin and collagen is difficult to follow. Dissolution of the fibrin and replacement of it by advancing granulation tissue is not seen.

The fibroblasts beneath the serosa and elsewhere likewise contain no *Rickettsiae*, and no organisms are found in fat cells, epidermis, smooth

muscle cells, striated muscle fibers, sebaceous or sweat gland cells or in the interstitial or epithelial cells of the testis and epididymis.

The reaction in the deeper tissues is primarily that characteristic of typhus in the human, namely a marked proliferation of capillary and lymphatic endothelium with occasional thrombus formation, and a marked perivascular accumulation of inflammatory cells, largely macrophages and cells of the lymphocytic series. The fibres of the cremasteric muscle undergo considerable necrosis and large numbers of polymorphonuclears accumulate about them. Frequently, also, there is degeneration of the parenchymal cells of the testis to a depth of 2 to 3 mm. with a definite purulent reaction. From the practically complete absence of *Rickettsiae* in these regions, we assume that the necrosis is caused by fairly strong toxins diffusing from the heavily infected serosal cells. The absence of gross necrosis in human tissues is probably due to the fact that only relatively small numbers of *Rickettsiae* are present in any given focus of infection.

After long search it is possible to find a few definite *Rickettsiae* in the true endothelial cells lining the capillaries and lymphatic vessels but organisms have never been found in the abundant cytoplasm of the perivascular macrophages or of the macrophages which collect in the loose areolar tissue beneath the serosa.

COMMENT

These observations lead us to conclude that in guinea pigs inoculated intraperitoneally infection with *Rickettsia prowazeki* is limited to the serosal cells *in situ* and the true capillary and lymphatic endothelial cells *in situ*. Their luxuriant growth in the serosal cells exactly parallels their behavior in the epithelial cells which line the gut of the louse. The very infrequent and scanty infection of the true endothelial cells is comparable to their behavior in human tissues. Genetically, mesothelium and endothelium are closely related, but structurally and functionally they differ markedly. The above observations seem to show that they differ also in their resistance to infection by the typhus organism.

The apparently complete absence of *Rickettsiae* from both blood and tissue macrophages in the exudate was rather surprising. In tissue cultures, the *Rickettsiae* of Rocky Mountain spotted fever are found in

phagocytic cells (Wolbach and Schlesinger (6)) but typhus *Rickettsiae* have not been shown to infect these cells. Experiments now in progress in this laboratory indicate that typhus *Rickettsiae* grow luxuriantly (for a time) in mesothelial cells *in vitro*, but are never found in phagocytic cells.

The above observations suggest very strongly that typhus *Rickettsiae* are likewise incapable of multiplying in fibroblasts. It is of course impossible to be sure that the fibroblasts in our material were adequately exposed to infection. Tissue culture work now in progress will, we hope, answer this question definitely.

Study of *Rickettsia prowazeki* in sections has strengthened its position as an obligatory intracellular parasite. In smears one often finds large numbers of diffusely scattered extracellular organisms, but in view of the observations made on sections it seems necessary to conclude that these organisms are spilled from cells which are ruptured in the process of making the smear.

The observation that the *Rickettsiae*-containing serosal cells are essentially non-phagocytic is in agreement with the recent views on the origin of the peritoneal phagocytes. Cappell (7) recently found the serosal cells slightly phagocytic for carmine particles after repeated injection and believed that they might give origin to a certain number of the peritoneal macrophages. The absence of *Rickettsiae* from the phagocytic cells and the absence of ink particles from the *Rickettsiae*-containing cells is regarded as evidence against the serosal origin of any important number of peritoneal phagocytes.

SUMMARY

1. A satisfactory method is described for the topographical study of *Rickettsia prowazeki* in sections of the scrotal sac of typhus-infected guinea pigs.

2. In such sections *Rickettsiae* are always intracellular.

3. Typhus *Rickettsiae* multiply luxuriantly in the serosal cells and produce great distention of these cells.

4. *Rickettsiae* may be found in small numbers in the endothelial cells lining the underlying capillaries in the testes and scrotum, but are not seen in perivascular macrophages, connective tissue cells, fat cells, smooth or striated muscle fibres or epithelial cells.

5. *Rickettsiae* are rarely phagocytosed in small numbers by polymorphonuclears but are never seen in lymphocytes, plasma cells or eosinophiles.

6. When infected mesothelial cells proliferate and desquamate, they rapidly lose their content of *Rickettsiae*.

7. The large mononuclear cells seen in smears of the scrotal sac exudate may be separated into two groups: (1) the serosal (mesothelial) cells which become heavily infected with *Rickettsiae* but which are not phagocytic for graphite ink and (2) the macrophages (phagocytic cells) which do not contain *Rickettsiae*.

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EXPLANATION OF PLATES

PLATE 22

FIG. 1. Drawing of a representative field from a Giemsa-stained smear of scrotal sac exudate after intraperitoneal injection of graphite ink. One serosal cell (lower left) is lightly infected with *Rickettsiae* but has ingested no ink particles. Several macrophages are present with ingested ink particles but without *Rickettsiae*. $\times 800$.

FIG. 2. Drawing of a representative field from a section of the scrotum and parietal tunica from a typhus-infected guinea pig. The majority of the serosal cells are heavily infected with *Rickettsiae*. Note the absence of *Rickettsiae* from the underlying cells and from the cells in the exudate. Regaud's fluid fixation. Giemsa stain. $\times 800$.

PLATE 23

FIG. 3. Photomicrograph showing a concentric whorl apparently formed by proliferation of serosal cells. Striated muscle of the scrotum is seen at the left. The row of cells occupying the position of the original serosa passes vertically through the center of the field. Note the *Rickettsiae* in these cells and the absence of *Rickettsiae* from the proliferated serosal cells at the right. $\times 789$.

FIG. 4. Higher magnification of a portion of the field shown in Fig. 3. Sub-serous collagen below. Layer of infected serosal cells crosses the center of the field horizontally. Proliferated serosal cells (without *Rickettsiae*) above. $\times 1578$.

(Pinkerton: Typhus fever. II)