SPOTTED FEVER

I. Intranuclear Rickettsiae in Spotted Fever Studied in Tissue Culture

By HENRY PINKERTON, M.D., and G. M. HASS, M.D.

(From the Department of Pathology, Harvard Medical School, Boston)

Plate 7

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The object of this paper is to describe an unusual morphological picture obtained by studying spotted fever infection by the tissue culture method.

Wolbach and Schlesinger (1) in 1923 cultivated tissues infected with *Dermacentroxenus rickettsi*. They maintained infectivity *in vitro* for 28 days and found the specific organism in increasing numbers, histologically, up to the 14th day. Their results indicated that definite multiplication of the organism had taken place, but the number of infected cells found histologically was relatively small.

In view of the voluminous multiplication of typhus *Rickettsiae* which we have obtained recently in tissue culture (2, 3), it seemed desirable to restudy spotted fever by the new technic.

The strain of spotted fever used in the experiments to be reported here was an atypical one, isolated in Minneapolis, Minnesota. We are indebted to Dr. H. A. Reimann for the opportunity of studying it. A description of the strain, including immunological evidence that it belongs in the spotted fever group, has been published by Reimann, Ulrich and Fisher (4).

This strain was chosen for tissue culture work because of the fact that guinea pigs reacting to it develop an acute inflammation of the scrotal sac, often resulting in a membraneous exudate similar to that found in endemic typhus. Cells infected with *Rickettsiae* are present in relatively small numbers in this exudate in the Reimann strain, but are probably more numerous than in fragments of infected testis and cremasteric muscle in typical Rocky Mountain spotted fever. In

the latter disease no exudate is present in the scrotal sac and one must rely upon the presence of *Rickettsiae* in the vascular lesions in the fragments of tissue used as explant material. Another advantage of this strain is that the presence of *Rickettsiae* can be easily confirmed by making smears of the exudate so that the most desirable material for setting out cultures may be selected.

Material and Methods

Membraneous exudate from the scrotal sac on the 2nd or 3rd day of fever was floated in Tyrode's solution, cut into small fragments and explanted, using the technic previously described for similar experiments with typhus *Rickettsiae* (2, 3). The cultures were incubated at 32°[1] and transferred usually every 6 or 7 days. The cultures were carried on for varying lengths of time ranging up to 32 days. At intervals certain cultures of each group were fixed and stained by the technic previously described and others were injected into guinea pigs for virulence tests. The cultures for injection, together with the fibrin clot in which they were embedded, were picked up with forceps and floated in 4 cc. of Tyrode's solution. taken up into a syringe with a needle of large calibre and injected intraperitoneally, They were then taken up into a syringe with a needle of large calibre and injected intraperitoneally, taking care in each case that the culture did not adhere to the sides of the syringe. (By holding the syringe against a dark background during injection it was possible to see the culture disappear through the proximal end of the needle.) The cultures for histologic study were fixed in Regaud's fluid and embedded in paraffin. Serial sections about 7 micra in thickness were stained by the Giemsa method.

RESULTS

The guinea pigs injected with cultures of various ages all reacted positively. It was noteworthy that a marked scrotal sac inflammation, resulting in palpable adhesions between visceral and parietal layers, occurred constantly in animals inoculated from the tissue cultures, whereas it was relatively rare in animals inoculated in the usual way with blood or scrotal sac exudate. This we ascribe to the higher concentration of *Rickettsiae* in the tissue cultures.

Large numbers of infected cells were found in paraffin sections of the cultures, and excellent morphological pictures of the intracellular parasites were obtained. In several cultures, more than half of the

[1] In a recent publication (5) it is shown that the temperature of incubation has a marked influence on the multiplication of typhus *Rickettsiae* in tissue culture.

cells present contained *Rickettsiae*. The organisms (as in the case of typhus) were confined to the interior of cells and never seen in the plasma clot except in very rare instances when cells had ruptured. They were never seen in the clot at a distance from the growing tissue.

The outstanding feature of the picture was the apparent predilection of these spotted fever *Rickettsiae* for the nuclei of the cells. In several preparations, the majority of infected cells showed only nuclear involvement. Infection of the cytoplasm without nuclear involvement was fairly common in all preparations, however. Cells in which both nuclei and cytoplasm were infected were quite rare. Within the nuclei the organisms were closely packed while in the cytoplasm they were almost invariably diffusely scattered. The majority of cells in which the cytoplasm was infected contained less than fifty *Rickettsiae*, and in no instance did the entire cytoplasmic volume become occupied by closely packed *Rickettsiae*.

Morphologically, the spotted fever *Rickettsiae* described here show great pleomorphism. The organisms range from minute coccoid bodies, about 0.20 μ in diameter to long thread forms (mostly chains of bacilli) up to 0.8 μ in diameter and 15 μ in length. The thread forms were much more frequent in the cytoplasm and the smaller bacillary and coccoid forms in the nucleus. Rarely, however, intranuclear rods ranging up to 4 μ in length were seen.

The organisms varied in their staining reaction from blue through the purples to red, depending on the degree of differentiation, but never attained quite the bright red color of the granules of polymorphonuclear leucocytes. In the cytoplasm they appear in some instances to retain the blue somewhat longer in the process of differentiation than did the fibrin clot in which the cells were embedded but the intranuclear forms were more markedly eosinophilic.

The various stages of intranuclear infection are shown in the accompanying illustration (Fig. 1). The initial stage is the appearance of from one to eight or more (most frequently one to three) small spherical groups of closely packed *Rickettsiae*, purplish red to bright red in color, and standing out clearly from the blue substance of the nucleus, even in undifferentiated sections. These clusters were composed of from three or four to fifty or more individual organisms. In the

smaller clusters, the rod-shaped organisms frequently formed a peripheral ring. A definite halo was always present about clusters of all sizes. In some instances the individual organisms of a group could not be resolved and the clusters appeared as hyaline masses (see upper left cell in Fig. 1). In the later stages, the clusters apparently become confluent and may occupy more than nine-tenths of the volume of the nucleus. There was invariably a pale clear ring at the periphery of the nucleus, however.

The nucleus itself was in some instances considerably swollen and often definitely wrinkled. In several cells the nuclear membrane appeared to have broken down so that nucleoplasm and cytoplasm had fused. In degenerating cells, clusters of *Rickettsiae* appeared as irregularly granular reddish purple structures, which, without study of intermediate stages, would not be accepted as microorganisms (see lower right cell in Fig. 1).

COMMENT

Wolbach (6) in 1919 found the specific organism of Rocky Mountain spotted fever massed within nuclei of epithelial cells in the gut, hypoderm and salivary gland of the tick. In spite of careful study by methods apparently adequate for its demonstration, it has never before been found in an intranuclear position in the tissues of any of its mammalian hosts. Its localization in nuclei in cultures of guinea pig tissues is therefore of considerable interest. The difference between the *in vivo* and *in vitro* pictures may depend on the lower temperature at which the tissue cultures were incubated. This question is being studied. We are also studying other strains of spotted fever by the same technic, since it seems improbable that the lesions described here are peculiar to the somewhat atypical strain used for this investigation.

The reason for the clustering of the organisms in nuclei and their diffuse distribution in the cytoplasm of cells is not apparent. Typhus *Rickettsiae* occasionally form separate groups in the cytoplasm of the cells in which they grow but these groups are rarely sharply outlined. The nucleus is less fluid than the cytoplasm and it seems possible that the aggregations of organisms may be comparable to bacterial colonies in solid artificial media.

The contrast between the behavior of spotted fever and typhus *Rickettsiae* in tissue culture is striking. The majority of cells infected with typhus *Rickettsiae* are filled almost to the point of bursting, but it is only the cytoplasm which is involved. In sections of such distended cells, the nucleus often stands out as a clear zone in the center of a dark purple mass of organisms. In spotted fever, it is the nucleus which becomes packed with organisms while the cytoplasm is only sparsely infected. Furthermore, spotted fever *Rickettsiae* are found within phagocytic cells (identified in the culture by their content of hemosiderin) while typhus *Rickettsiae* are strictly confined to non-phagocytic cells (7, 2).

Morphologically, the two organisms appear very similar when studied by this method. In size and shape they are practically identical. The spotted fever organisms in the cytoplasm are more frequently stained blue than the typhus organisms, but this is not true of the intranuclear forms.

It seems necessary to comment on the resemblance of the intranuclear clusters of *Rickettsiae*, especially when imperfectly fixed and stained, to certain of the structures of unknown nature found within cells in the so called virus diseases. The concept that some of these structures are masses of organisms has been expressed from time to time and in recent years has been especially sponsored by Goodpasture (8). Cowdry and Kitchen (9), however, on the basis of the lack of iron and thymonucleic acid and the intense acidophilic properties of the inclusion bodies believe that they are the result of chemical and physical changes.

The etiology of spotted fever has been thoroughly cleared up and this disease has been definitely taken out of the heterogeneous group of virus diseases. It is similar to virus diseases, however, in the definite association of infectivity with cells rather than with body fluids, and in the fact that the etiologic agent has not been made to multiply in artificial media in the absence of living cells.

The lesion described here is the first instance in which a definite microorganism has been shown to be parasitic in clusters in the nuclei of mammalian tissues. It therefore seems not improbable that some of the unresolved intranuclear structures now classed as inclusion bodies may be of a similar nature.

SUMMARY

Spotted fever infection has been studied in tissue cultures grown at 32°C. The behavior of spotted fever *Rickettsiae* is compared and contrasted with that of typhus *Rickettsiae* under similar conditions. The spotted fever organisms multiply extensively in the nuclei of cells where they form spherical clusters of various sizes. These structures are compared and contrasted with intranuclear inclusion bodies.

Note.—After preparing this paper for publication, similar results were obtained from the strain of "Eastern spotted fever" isolated by Rumreich, Dyer and Badger (10). Cultures from guinea pigs reacting to this strain were set out both from the scrotal sac and from the spleen. In both cases the cultures became heavily infected with *Rickettsiae* on the 11th and 18th days and the organisms were massed within nuclei in this strain also. This is evidence of the essential similarity of the two strains, and additional evidence that both belong in the spotted fever group.

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EXPLANATION OF PLATE 7

FIG. 1. All cells are from a single paraffin section of a tissue culture of infected scrotal sac exudate from a guinea pig reacting to spotted fever. Five of the cells are from a single field, while the others have been selected from other parts of the section to show more completely the morphological range. The ratio of cytoplasmic to nuclear infection is about the same as that in the section as a whole. The cultures were fixed in Regaud's fluid and embedded in paraffin. Serial sections about 7 micra in thickness were stained by the Giemsa method. ×1500.

Note especially the cells in the upper left and lower right corners, in which individual organisms cannot be resolved. The resemblance of the structures in these cells to inclusion bodies is striking.

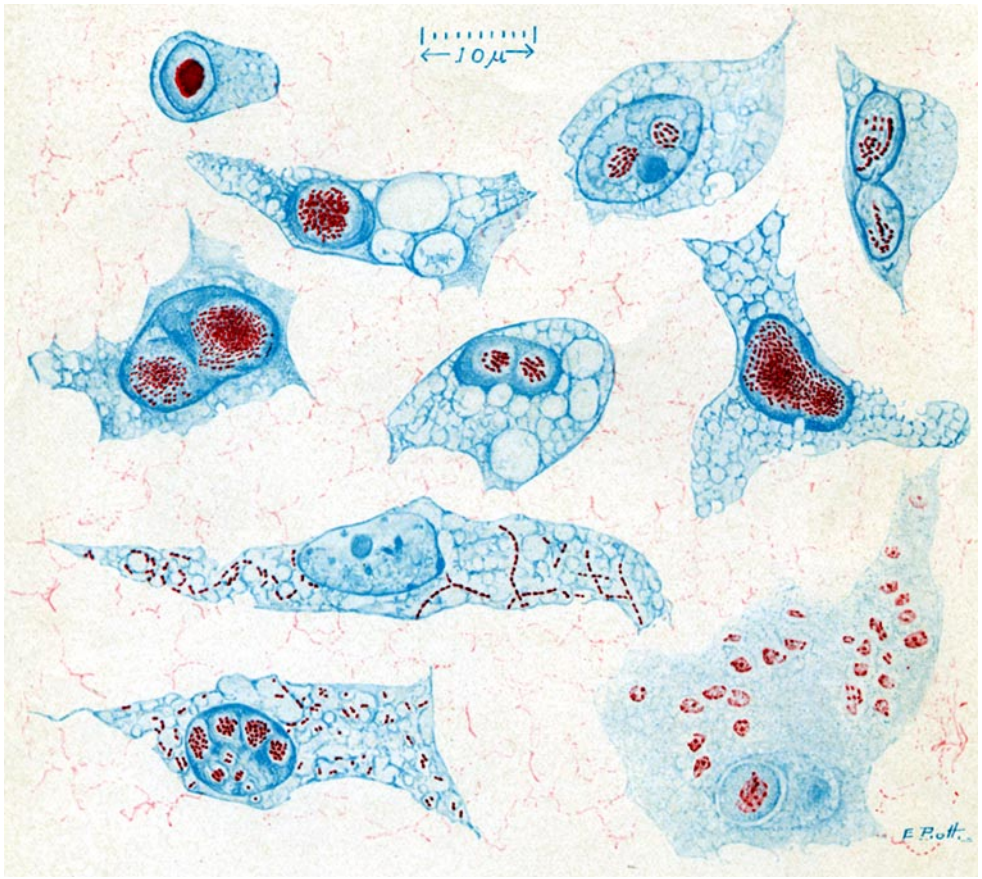

FIG. 1

(Pinkerton and Hass: Spotted fever. I)