AN EXPERIMENTAL STUDY OF THE HISTOGENESIS
OF THE MILIARY TUBERCLE IN VITALLY
STAINED RABBITS.*

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Plates 36 to 39.

INTRODUCTION.

The inadequacy of the methods hitherto applied to the study of
the histogenesis of the tubercle led us to produce tuberculosis experi-
mentally in vitally stained rabbits. In rabbits injected with trypan
blue the Kupffer cells stain deeply, while the blood cells are free from
stain, and we thought that this might throw some light on the part
played by the Kupffer cells in the formation of the miliary tubercle
in the liver.

HISTORICAL. 1

Opinion is still divided as to the origin and structure of the miliary tubercle. About fifty years have elapsed since Virchow first demonstrated the tubercle, named it, and showed that it was the distinctive product of tuberculosis. Although Virchow saw little in its morphology save round cells, it was not long before Langhans found the giant cell and noted its practically constant occurrence. Schueppel discriminated the three cell types (lymphocytes, epithelioid cells, and giant cells) which we recognize to-day as being usually present, if not of invari-
able occurrence, and of valuable diagnostic aid. The typical arrangement or suc-
cession of these three cell types has come to be a law (Ziegler).

Though we are now aware that they are by no means pathonomical for the
tubercle, nevertheless the giant and epithelioid cells are peculiar structures, iden-
tical with none of the familiar fixed cells of the body, although descended from
them. Their manner of descent and transformation have consequently claimed

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1 The literature has been reviewed by Wechsberg, Dürck, Dürck and Ober-
dorfer, and others, and only the more important views will therefore be given.
much interest. After the discovery by Koch in 1884 of the etiological agent of tuberculosis, many investigators began to study the formation of the tubercle after the injection of pure cultures. One view identified with the name of Metchnikoff,\(^2\) the other with that of Baumgarten, might be said to represent the views of the French and German schools respectively. Metchnikoff maintains that the tubercle is of leucocytic origin, and Baumgarten believes that the fixed tissue cells alone are responsible for its formation.

Metchnikoff's contention must be regarded as the natural outcome of his theory of the cells concerned everywhere in the defense of the body against bacterial disease. To the chief cells he has applied the term macrophage, meaning thereby a class of large mononuclear cells doubtless identified by others as wandering cells, which Metchnikoff and his followers considered to have arisen from the overgrowth of true lymphocytes. Stages in this transformation were described, so that there was no difficulty in imagining that with the increase in cell body, the deeply-staining lymphocytic nucleus became the large clear nucleus, poor in chromatin, of the epithelioid or giant cell. The phagocytic properties of the latter, however, were the chief cause of Metchnikoff's derivation of them from his macrophage, and he emphasized the almost invariable existence of bacilli in these cells. Unquestionably the convictions of Metchnikoff came more as a general result of his notions of the cells concerned in immunity than as the result of an exhaustive morphological research, but it is interesting to find that Koch himself was disposed to regard wandering cells as the chief elements in the tubercle, even though he imagined that their subsequent death might result in the participation of various fixed cells.

Baumgarten, and many investigators after him, have established the fact that the tubercle is not merely a heaping up of preformed elements, but that active proliferative processes are always concerned in its formation. The evidence for this was clear, for frequent mitoses are found, and inasmuch as these affect the chief cells of the immediate neighborhood, regardless of where the tubercle lies, Baumgarten and others have concluded that these cells alone are essential in tubercle formation, and that the most various tissue elements, including those as far apart as connective tissue and epithelium, could play this part, but that leucocytes could not. Indeed Baumgarten denied any but the latest and most trivial participation of leucocytes in the actual beginning of the tubercle, though subsequent investigators among his own countrymen could not, of course, confirm this. The preliminary outpouring of polymorphous cells and secondary lymphocytic infiltration which the French school had so clearly seen were undeniable, though Baumgarten's adherents denied a wider interpretation of this phenomenon as completely as the French school had denied the interpretation put on mitoses, the presence of which they also admitted. According to Baumgarten's theory, Kostentsch and Wolkow describe the inception of a tubercle as follows: (1) the formation of a serofibrinous exudate, (2) migration of polynuclear leucocytes which rapidly disintegrate, (3) proliferation of fixed tissue cells to form epithelioid cells, (4) migration of mononuclear leucocytes which take a peripheral

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\(^2\)Metchnikoff's views are upheld by Yersin, Borrel, Leray, and Wallgren; Baumgarten's by Klebs, Kockel, Kostentsch and Wolkow, Wechsberg, Miller, Watanabe, Oppenheimer, Straus, Morel, and others.
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position around the epithelioid cells, and (5) secondary migration of polynuclear elements when the tubercle degenerates.

The question of the origin of the cells forming the tubercle depends on our ability to distinguish sharply cell types. Few reliable criteria have been advanced for such a discrimination, although the recent work of Wallgren and Oppenheimer should be mentioned. These investigators have taken advantage with us of the fact that the production of tubercles within the liver lobule permits us to work in a relatively simple territory, where besides the blood cells and endothelium of the hepatic sinuses, only the liver cells occur, all the connective tissue elements, for instance, being absent.

Oppenheimer, starting from the fact that silver particles are phagocytized largely by Kupffer cells, has produced hepatic tubercles after collargol injections. The tubercles which appeared after the transitory leucocytosis were composed of silver-containing epithelioid and giant cells, and Oppenheimer naturally derived these from the endothelium. It is unfortunate that the conclusiveness of his experiments is open to doubt on the grounds that normally some of the blood cells engulf the silver particles. Furthermore, Wallgren, also working with rabbit liver, has reached an opposite conclusion, and substantiates fully the lymphocytic origin of the cells. There appears to be sufficient reason, therefore, for a reinvestigation of the relatively simple liver tubercle with a more decisive method of study.

MATERIAL AND METHODS.

In our experiments rabbits were used exclusively. The animals were vitally stained by repeated intravenous injection of a freshly prepared aqueous solution of trypan blue. The dye is a benzidin of the following formula,

\[
\begin{align*}
\text{NH}_2\text{OH} & \quad \text{OH} \\
\text{NaO}_3\text{S} & \quad \text{CH}_2
\end{align*}
\]

and has been used as a vital stain, by Bouffard, Goldmann, Schulemann, Evans, and others. The staining in the experiments to be recorded may be divided into two groups: the acute staining, where daily intravenous injections of twenty cubic centimeters of 1 per cent. solution of the dye were given during a few days, and which resulted chiefly in a pigmentation of the Kupffer cells; and the chronic staining where the injections were made at longer intervals and over a longer period of time. In these animals, besides the staining of the Kupffer cells, the blood of the liver capillaries contains many vitally stained macrophages. Both types of staining are adaptable to the study of the histogenesis of the tubercle, and the method of staining will be briefly given in each experiment.
Emulsions of bovine tubercle bacilli were made in the usual manner. The bacteria were carefully removed from the glycerin bouillon medium, dried on sterile filter paper, ground, and taken up in normal salt solution. This was then put into a shaking machine for two hours. The strength of the suspension was graded so that each cubic centimeter contained one milligram of dried tubercle bacilli.

The animals were anesthetized, a loop of bowel was exposed aseptically, and ten cubic centimeters of the suspension of tubercle bacilli were injected into a small radical of the mesenteric vein. The animals were killed at varying intervals, from one half hour to eleven days, and immediately injected through the ascending aorta, by pressure, with a 10 per cent. solution of formalin. Under these conditions the tissues are rapidly fixed throughout, the intra-acinar capillaries are dilated, and most of their blood content is washed out. The liver was then removed, cut into small strips a few millimeters in thickness, and replaced in formalin. Satisfactory results were obtained from frozen sections, and these were used throughout the study. With the above fixation, sections five micra in thickness were readily obtained. The sections were stained with aqueous cochineal, which contrasted well with the blue vital stain. Other sections were stained in Ziehl-Nielson carbol fuchsin for five minutes, decolorized in 10 per cent. hydrochloric acid alcohol, thoroughly washed in distilled water, and counterstained with Delafield's hematoxylin. Weigert's fibrin stain was used in some of the early stages.

THE RESULT OF THE INJECTION OF VITAL STAIN UPON THE LIVER OF NORMAL ANIMALS.

The literature on the histology of the normal rabbit's liver has been reviewed recently by Wallgren. Concerning the Kupffer cells, Wallgren quotes Schilling who considers them to be endothelial phagocytes and functional stages of liver endothelium. He believes that they arise from normal liver endothelium, by swelling and paling of their nuclei, and that probably every endothelial cell passes through this change from time to time.

In order to check Schilling’s results, we killed an animal and in-
jected formalin through the aorta. The liver cells occasionally contain two nuclei, and a mitosis may occasionally be found. The nuclei of the endothelial cells of the capillaries are relatively wide apart, and two are found opposite each other only in rare cases. They are closely approximated to the columns of liver cells, and vary considerably in appearance. Many of them are flat, like ordinary endothelium, and there are transition forms to the larger ones with vesicular nuclei that bulge into the lumen of the capillary. The larger ones rarely contain an ingested polynuclear leucocyte or a granule of brown pigment.

EXPERIMENTAL PART.

VITALLY STAINED NORMAL ANIMALS.

Rabbit 1.—Mar. 14, 1912, 9 A.M. Injected with 20 c.c. of a 1 per cent. aqueous solution of dye in ear vein.

Mar. 15, 9 A.M. Killed. Fixed with formalin through aorta.

Liver.—The liver cells contain a few granules of trypan blue. The Kupffer cells are about normal in size and occurrence. Most of them contain a varying number of small blue granules. Endothelial cells with two nuclei or cells in mitosis are not found. Phagocytosis of polynuclear leucocytes or red blood cells is uncommon.

Blood.—The usual white blood cells, including lymphocytes and mononuclear and polynuclear leucocytes, occur and are unstained. Besides these large cells with clear non-granular protoplasm, vesicular nuclei and definite nucleoli occur. The nucleus may be round, oval, or bent. The nature of the cells is not always determinable. They appear to be similar to the large lymphocyte or polyblast emphasized by Wallgren.

Interstitial Tissue.—Normal; no vitally stained cells.

Rabbit 97 A.—Mar. 10 and 11. Intravenous injection of 20 c.c. of 1 per cent. solution of dye. Killed at the end of forty-eight hours.

Liver.—The cells contain a few small granules of vital stain. The Kupffer cells are more numerous, and many of them are increased in size. The majority contain blue granules of irregular sizes. These may be dense, or there may be only a few granules scattered near the nucleus, or even towards the tip of one of the protoplasmic processes of the cell. The dye is more abundant in the larger cells though the flat ones may contain a few granules. As the cell becomes larger, the nucleus becomes more vesicular, and rarely two nuclei may be found within one cell body. One mitotic figure was found. Phagocytosis occurs as usual.

Blood.—There are no vitally stained cells in the capillaries. The blood picture is similar to that of rabbit 1.

Interstitial Tissue.—An occasional clump of blue granules is found in the periportal tissue; but whether these are contained within the endothelium of the vessels or in the wandering cells cannot be determined.

Rabbit 200.—April 13, 14, 15, and 16. Intravenous injection of 20 c.c. of 1 per cent. solution of dye. Killed at the end of the fourth day.
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Liver.—The cells contain definite, large, bright blue granules of dye, and may have two nuclei. The Kupffer cells are conspicuous. They are definitely increased in numbers and most of them are large and heavily laden with varying sized granules of dye. Some of the enlarged Kupffer cells are no longer firmly attached to the liver columns, and may be free in the lumen with their processes extending to the column of the liver cells. These cells may be cut in such a way as to appear round and free in the capillary. Not only are the Kupffer cells enlarged, but an occasional one with two or three nuclei and a rare mitotic figure may occur. In the latter the pigment granules are never found in the spindle. The nuclei of the large Kupffer cells may be irregular, and assume a polymorphous shape. The phagocytic property of the large Kupffer cells is demonstrated by the frequent finding of polymorphonuclear leucocytes or red blood cells within their bodies.

Blood.—The injection of formalin through the aorta was unsuccessful, and as a consequence, considerable blood is contained within the vessels. The larger veins contain normal unpigmented blood elements. The larger polyblast-like cells occasionally contain a few very fine granules of dye.

Interstitial Tissue.—There is an occasional pigmented cell, the nature of which could not be definitely determined.

Rabbit 80.—Six successive doses of 20 c.c. of 1 per cent. solution of dye were given on six successive days. The animal died shortly after the last injection, i.e., early on the sixth day. The findings were similar to those in rabbit 200, except that two large pigmented giant cells were found in the liver.

Rabbit 49.—Nov. 23, 27, Dec. 6, 31, Jan. 5 and 13. 103 c.c. of 1 per cent. aqueous solution of trypan blue were given in approximately 20 c.c. doses. Jan. 19. Animal killed by a blow on the head, and 500 c.c. of 10 per cent. formalin injected through the aorta at a pressure of 80 mm. of mercury.

Liver.—The cells show a few granules of dye. Cells with two nuclei and occasional mitoses in the liver cells may be found. The Kupffer cells are conspicuous on account of their increased size and number, and their content of pigment granules. Kupffer cells with two or more nuclei are common and many large giant cells with innumerable nuclei are found. Frequently they occupy the position of the normal Kupffer cells, but sometimes they appear to be free. Mitosis is never demonstrable in them, but all transitions occur from those with two, three, and even more nuclei. The nuclei of the cells are large and twisted, so that they have a polymorphous appearance, and not infrequently the lobes may be connected by delicate threads of nuclear material. Phagocytosis is seen as usual.

Blood.—Besides the elements found in the previous cases, occasional large macrophages occur, often so densely pigmented that the nature of the cell is entirely obscured.

Interstitial Tissue.—The same as in the previous cases.

From the foregoing experiments it is evident that following successive intravenous injections of trypan blue the Kupffer cells become laden with the dye, enlarge, and increase in number, and finally form large free syncytial masses or giant cells containing one or
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several nuclei. The endothelial cells which undergo this transformation and finally become large free macrophages are filled with increasing numbers of the vital blue granules in their cytoplasm. The blood cells, on the contrary, remain free from the dye and undergo no noticeable changes. It is important to note that whereas the dye thus stimulates the formation of free endothelial cells, it does so only after an interval of four or five days; within this interval only mild proliferative changes occur, resulting merely in the enlargement of Kupffer cells and their multiplication in situ in the vessel wall. We shall find in tuberculosis a far more rapidly changing picture.

EXPERIMENTAL TUBERCULOSIS IN UNSTAINED RABBITS.

Rabbit Ioz.—Killed forty-eight hours after inoculation with a suspension of 10 mg. of bovine tubercle bacilli in superior mesenteric vein. Dense cellular masses are scattered through the liver, either in the region of the periportal vessels or in the intra-acinar capillaries.

Liver.—The cells are coarsely granular and vacuolated, but otherwise present nothing of note. The Kupffer cells are more numerous than in the normal liver, and vary in size. Many of them are much larger, and as they increase in size the nuclei become more vesicular. The smaller, more slender Kupffer cells lie against the column of the liver cells like an endothelial membrane, while the larger ones seem to be less firmly attached and may be seen almost free in the lumina of the capillaries. Here their contour is easily made out. They are spindle- or winged-shaped, with long processes that often cross the bed of the capillary before they approximate themselves to the wall of the liver columns. When cut at different angles they often resemble polyblasts. They may contain polymorphonuclear leucocytes, which in many instances are well preserved and appear to be as healthy as those free in the capillaries. Around these ingested cells is a clear vacuolar space, which may cause an indentation in the nucleus where the latter comes in contact with the ingested cell. The ingested cell gradually undergoes disintegration. The chromatin may first disappear and leave a pale homogeneously staining protoplasm containing only a few chromatin specks, or the protoplasm may be digested or shrunken, leaving a small structureless mass of chromatin. Mitotic figures occur abundantly in the Kupffer cells, and Kupffer cells with two or three nuclei are found.

Blood.—The capillaries are dilated. Here and there in the smaller capillaries a few red blood cells or a polymuclear cell may be seen. Mononuclear cells also occur, some of which are small lymphocytes. By far the greater number, however, are irregular round or oval cells whose nuclei present no constant picture. They are apparently quite active, and while some have the chromatin arranged wheel-like, others are in division. Some of the cells are definitely Kupffer cells, since they are still attached by one or more processes to the columns of liver cells. Others are not in the capillaries, but occur as small nests of cells in
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indentations between the liver cell and the endothelial lining. In one such area the Kupffer cell may be seen sending a process down between the liver cells. The larger clumps or plugs are in the periportal vessels, whose endothelium is swollen and partly desquamated. The lumen contains, besides these desquamated cells, one or more giant cells, polyblastic leucocytes, and mononuclear cells. One nodule, for instance, has a large, crescentic, multinucleated giant cell at one pole. The nuclei of this cell form a horseshoe, and several small beaded bacteria in the protoplasm occur. The remainder of the nodule is composed of numerous stellate spindle and oval cells with pale vesicular nuclei. Two are dividing. The vessel wall is not clearly distinguishable, and the cellular accumulation is present to a slight extent in the surrounding tissue. The giant cells, Kupffer cells, and spindle or oval cells contain the bacteria, which occur occasionally in polyblastic leucocytes also.

In some areas pictures occur which suggest the possibility that these giant cells may increase in size by fusion. Cells with nucleus and protoplasm similar to that of the giant cell are found in close apposition to each other.

Interstitial Tissue.—In the region of the periportal spaces the interstitial tissue is obscured on account of the frequent localization of the clumps in these areas, described above. Fibrin could not be demonstrated in the clumps.

Rabbit 84.—Killed seven days after inoculation.

Liver.—The section shows the liver tissue to be infiltrated with large nodules which tend to become confluent. The cells show nothing of note except atrophy where the columns are pressed apart by the intravascular nodules. Kupffer cells are numerous. Many of them are large and multinucleated. Mitosis is frequent and was found in one cell with three nuclei. Cells may be seen dividing with the line of division perpendicular to the long axis of the vessel. Some of the Kupffer cells are more or less rounded and indistinguishable from the polyblasts in the capillaries except by their processes.

Blood.—The cell picture resembles that found in the previous experiment. Polyblastic-like cells are abundant and many show mitosis. They are also found in nests beneath the endothelium.

Plugs.—The smaller plugs have a central giant cell surrounded by Kupffer cells and large round cells. The larger nodules show central caseation with a peripheral zone composed of the above cell types.

Bacteria are abundant and occur exclusively within the bodies of cells. The giant cells and particularly the Kupffer cells contain the organisms.

Summary.—The bacteria at the end of forty-eight hours are all intracellular, and frequent degenerative forms are encountered. The polyblastic leucocytes rarely contain tubercle bacilli, while the giant cells, Kupffer cells, and mononuclear cells contain them in the order named.

The Kupffer cells apparently are active. Frequent mitoses are found in them and Kupffer cells with two or more nuclei occur. These may form giant cells which may either occupy their normal
position or lie free in the lumen of the capillary. The giant cells
do not show mitoses, but their nuclei are tortuous and polymorphous
suggesting multiplication by budding. Elsewhere, within the cen-
ter of tubercles pictures are occasionally encountered which suggest
the possible formation of giant cells, or rather their increase in size
by fusion, but these pictures are rare and not convincing.

Besides polynuclear leucocytes and definite lymphocytes in the
capillaries, other large white cells occur which are difficult to class-
ify. These cells are round or oval with homogeneously staining
protoplasm and varying types of nuclei. They frequently show
division. They are probably the cells spoken of as polyblasts by
Wallgren. Some of them have streamers that extend to a neighbor-
boring Kupffer cell; some seem to be attached to the Kupffer cells,
but the majority are free in the capillaries. Cells exactly like these
may be found singly, or in nests lying in hollows of the liver cells
below the endothelium. Individual cells of such a nest may be in
mitosis. The Kupffer cell overlying the nest may also be in mitosis.
The similarity between the cells of the nests and the large unclassi-
fied cells in the liver capillaries on the one hand, and the similarity
and anatomical relation of both types to the dividing young Kupffer
cells on the other hand are striking.

The nodules are of two types; those occupying the larger portal
venules, and those of the intra-acinar capillaries. The first, even
at the end of seven days, contain polynuclear leucocytes, though
they are much less numerous than at the end of forty-eight hours.
Besides these, large giant cells, stellate cells resembling Kupffer
cells, and large mononuclear cells, compose the clumps. The vessel
wall may or may not be intact in any one nodule. Outside the ves-
sel similar cells may sometimes be found. Fibrin is not demon-
strable.

The smaller intra-acinar nodules afford the more satisfactory
study, consisting as they do of giant cells, which may or may not
preserve their normal relation to the lining of the capillaries. Often
the giant cell is pushed out and surrounded on all sides by cells
similar to Kupffer cells, and by large mononuclear cells which may
show mitosis, and occasionally by lymphocytes. These nodules com-
pletely fill the containing vessel, and in the later stages the centers
may be caseous.
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The difficulty of determining the origin of the cells of the young tubercle from ordinary stains is evident from the above description. It is impossible to say what the origin may be, beyond the fact that mononuclear cells are involved. Whether the endothelial or the mononuclear blood elements are concerned cannot be decided. An intermingling of the two cell types has taken place, and every transition from either type to the giant cell can be imagined. The vital stain, free from any possibility of inclusion of the blood cells, should furnish discriminating light here.

Rabbit 98.—Killed twenty-four hours after intravenous injection of 20 c.c. of 1 per cent. solution of trypan blue, and one half hour after inoculation with tubercle bacilli.

Liver.—Aside from slight pigmentation, the cells show nothing abnormal. The Kupffer cells are enlarged and relatively deeply stained. They correspond in size and intensity of vital staining to those of rabbit 97 A. Mitotic figures are found in much greater frequency than in the animals that received vital stain alone, and Kupffer cells containing two to three or four nuclei, though rare, may be found. The Kupffer cells show phagocytosis.

Blood.—Except for an apparent increase of polymuclear leucocytes there is no change in the blood picture.

Plugs.—Both in the larger interlobular veins and in the intra-acinar capillaries cell accumulations occur. The walls of the veins may be broken and their fibers stained vitally. Within the vein a bluish fibrillar substance occurs, in which many polymuclear leucocytes, a few red blood cells, and an occasional lymphocyte are enmeshed. In the center of these areas clumps of bacteria are frequently found. The surrounding polymuclear cells seldom contain bacteria, but the Kupffer cells frequently contain organisms even in distant areas. These cells may show mitosis, and a small group of cells may accumulate around them. Fibrin is not demonstrable.

Rabbit 100.—Killed thirty hours after intravenous injection of 20 c.c. of 1 per cent. solution of trypan blue, and six hours after inoculation with tubercle bacilli. Clumps of blue staining material scattered throughout the liver are readily seen with the naked eye.

Liver.—The cells contain granules of trypan blue. Many of the Kupffer cells are enlarged. Granules of vital stain are found in them as well as in the normal endothelial cells, but a number of cells are still unstained. Kupffer cells with several distinct nuclei occur occasionally. These may be so large that they almost occlude the lumen of the capillary. Mitoses occur in the Kupffer cells, but are not demonstrable in those with more than one nucleus. Phagocytosis is marked.

Blood.—The capillaries contain little blood. Here and there a few red blood cells occur. Polynuclears may also be seen, but they are not abundant. Some

*This animal was not injected with formalin after death on account of the risk of washing out the fresh capillary plugs in the liver.
of the mononuclear cells are definite lymphocytes, but others resemble polyblasts. None of the blood cells are pigmented with the vital stain.

**Plugs.**—Most of the plugs are small and lie in the intra-acinar capillaries which they may entirely occlude. They consist of large, swollen Kupffer cells with varying numbers of large vesicular nuclei, a few polymorphonuclears, and an occasional mononuclear cell. The Kupffer cells are easily identified by their shape and pigment content. Some of them may be still attached to the vessel wall. They may contain bacteria, but this is difficult to demonstrate, since the bacteria are obscured by the vital stain. Here and there a mass of blue staining rods which resemble the bacteria occur. The larger plugs resemble those in the half hour stage.

**Rabbit 97 B.**—Mar. 10. 20 c.c. of trypan blue injected into ear vein.

Mar. 11. 20 c.c. of trypan blue injected in ear vein and inoculated with tubercle bacilli.


Scattered through the liver tissue are plugs of material conspicuous on account of their blue stain.

**Liver.**—The cells are normal in appearance and contain granules of trypan blue. The Kupffer cells are increased in size and number and contain many blue granules. The number of granules varies; some have only a few small ones, while others are so densely packed with them that the nucleus is obscured. Mitotic figures occur (figures 1, 2, and 3), and multinucleated cells are much more abundant than in the six hour stage. Phagocytosis is marked.

**Blood.**—The capillaries are dilated, but contain little blood. Polynuclears and red blood cells occur as usual. Mononuclears are more abundant. Most of them resemble polyblasts, but some are evidently Kupffer cells cut tangentially, and still have a process extending to the wall of the capillary. These have blue granules. Still others are found similar to them in every respect, but not attached to the vessel wall and without pigment granules. These cells are occasionally found lying in indentations of the liver cells below the Kupffer cells. With the exception of one polymorphonuclear leucocyte containing a few pigment granules, the cells of the circulating blood are normal.

**Plugs.**—These stand out sharply on account of the concentration of vital stain in them. Some of them consist of a single giant cell which is sometimes still attached to the vessel wall, and surrounded by only a few stellate Kupffer cells well laden with pigment granules. The larger plugs show a center of bluish material, in which polymuclear leucocytes and mononuclear cells are found. Surrounding these one or more pigmented Kupffer cells, or mononuclear, polyblast-like cells occur and form the remaining constituents of the plugs.

**Bacteria.**—A few bacteria are found in the Kupffer cells and in the giant cells. They are, however, made out with difficulty, as they are either stained or overshadowed by the trypan blue.

**Rabbit 92.**—Mar. 1. Intravenous injection of 21 c.c. of trypan blue.

Mar. 2. Intravenous injection of 21 c.c. of trypan blue. Inoculated with tubercle bacilli two hours later.

Mar. 3. Intravenous injection of 21 c.c. of trypan blue. Animal killed thirty-six hours after inoculation.

**Liver.**—The section is studded with many masses of vitally stained cells,
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usually near the periportal spaces. The liver cells are coarsely granular and vacuolated. They contain a few small granules of trypan blue. The Kupffer cells are almost all enlarged and appear more numerous. The greater number of them are stained as usual.

A considerable number of cells with two, three, or more nuclei are found. These may still occupy their positions on the capillary lining. Giant cell formation is particularly marked (figures 5, 6, and 7). Mitoses are found in unusual numbers in Kupffer cells with one nucleus, but not in multinucleated cells.

Blood.—The capillaries contain red blood cells, and polymuclear leucocytes, but the mononuclear cells predominate. Some of them are lymphocytes, but the majority are similar to polyblasts. The latter are not infrequently seen in mitosis.

Plugs.—More numerous than in rabbit 97 B. In the larger ones, the polymuclear leucocytes have to a great extent disappeared. Deeply pigmented giant cells still partially attached to the vessel wall, stellate pigmented and non-pigmented Kupffer cells, and polyblast-like cells occur as usual. The polyblast-like cells in some places seem to push the giant cell away from its attachment to the vessel wall and infiltrate the surrounding tissue to a slight extent. Mitoses occur in these polyblast-like cells and are often demonstrable in the Kupffer cells in the vicinity of the larger plugs. The smaller intra-acinar plugs present the usual picture. They invariably contain a large pigmented multinucleated giant Kupffer cell (figure 8), and in some instances are surrounded only by irregularly pigmented Kupffer cells. The Kupffer cells vary in the amount of their pigment. In other instances polyblast-like cells are associated with the Kupffer cells at the periphery of the giant cells. These cells are not pigmented but sometimes cells almost identical in type contain a few granules. The smaller plugs may entirely occlude the capillary, and in some instances, where the giant cell is still attached in its original position, the lumen of the vessel may be entirely obstructed by it alone.

Bacilli.—Acid-fast involution forms occur rarely in the giant and Kupffer cells, but they are demonstrated with difficulty.

Rabbits 95 and 96.—Mar. 1. Intravenous injection of 21 c.c. of trypan blue.
Mar. 2. Intravenous injection of 21 c.c. of trypan blue, and 10 mg. of tubercle bacilli.
Mar. 3. Intravenous injection of 20 c.c. of trypan blue.
Mar. 4. Killed fifty-four hours after inoculation.
A detailed description of the cases will be omitted. Only the points will be emphasized which have not been noted in the previous experiments.

Liver.—The Kupffer cells show the usual pigmentation, mitosis, phagocytosis, and giant cell formation. One multinucleated Kupffer cell was found in which one of nuclei was in mitosis.

Blood.—The usual cellular elements are present. Polyblastic cells are the most conspicuous. They occasionally show mitosis, and some of them contain a few minute pigment granules. Cells similar to these, but more pigmented, occur singly or in nests in small pockets formed between the Kupffer cells and the liver cells. Individual cells in the nests may be undergoing mitosis. Their direct origin from Kupffer cells was not demonstrable, although large Kupffer cells in their vicinity, in some cases lying over the nest, were seen to be undergoing mitosis.

Plugs.—The larger plugs contain fewer polymuclear leucocytes; the smaller
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"ones are more cellular, otherwise they resemble those described in the previous experiment. In rare instances pigmented Kupffer cells may be so closely packed against a giant cell that the possibility is suggested of a giant cell increasing in size by fusion with Kupffer cells. In one tubercle occurring in a large periportal vein a tubular structure was found lined by swollen, pigmented Kupffer cells; at one end the cells dilated and filled the lumen, giving the appearance of a giant cell.

Rabbit 74.—Jan. 16. Intravenous injection of 14 c.c. of trypan blue.
Jan. 17. Intravenous injection of 20.5 c.c. of trypan blue.
Jan. 18. Intravenous injection of 20.5 c.c. of trypan blue and 10 mg. of tubercle bacilli.
Jan. 19. Intravenous injection of 20.5 c.c. of trypan blue.
Jan. 20. Intravenous injection of 20.5 c.c. of trypan blue.
Jan. 21. Killed seventy-two hours after inoculation with tubercle bacilli. The giant cells are particularly numerous and stained brilliantly with the vital stain. They are so often attached to the vessel wall that where this is not found it may be attributed to the plane of the section. One giant cell had three nuclei, one of which was undergoing mitosis. The polymorphonuclear leucocytes in the circulating blood rarely contain a few blue granules. Kupffer cells are found free in the capillaries; they may retain their usual shape, or may be rounded with only a single process connecting them with the capillary lining. Such cells may be pigmented, and are hence distinguishable from the polyblastic cells.

Plugs.—In one of the plugs a non-pigmented giant cell was found. Pigmented and non-pigmented Kupffer cells, indistinguishable from polyblasts, may be found free in the capillaries. They may have several nuclei and the folding of the nucleus suggests an increase by budding. It is possible that these Kupffer cells are young, and the transition forms suggest that they may be the source of non-pigmented giant cells.

Experiments were carried on similar to those described in which the animals were killed at varying intervals up to eleven days. No new facts were brought out, however, and consequently a detailed description will not be given.

Rabbit 44.—A chronically stained animal had received the usual dose of trypan blue at regular intervals from Nov. 21 to Feb. 9, 160 c.c. of dye being injected during this period. On Feb. 15 the animal was inoculated with tubercle bacilli and on Feb. 19 a lobe of the liver was excised for study.

Liver.—The cells show the usual picture found in chronically stained animals. Large numbers of deeply stained macrophages occur throughout the liver capillaries. Definite tubercles were found in considerable numbers, usually located near the periportal areas. These are composed of central giant cells, and epithelioid and mononuclear cells. Occasional polymorphonuclear leucocytes are found in the larger clumps. The point of chief interest, however, is that the tubercles contain practically no vital stain. The small amount of stain that is found in the tubercle appears as fine granules in the giant and epithelioid cells.

This case (rabbit 44) seems to us of interest from two points of view. First, the contention that preexisting wandering cells form the tubercle is decisively disproven. There existed in this animal a great number of large free wandering cells electively stained so that their participation in the tubercle would have given us stained cells there. Secondly, the experiment demonstrates that the vital dye must be present in quantity in the body fluids so as to be accessible to
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the new growth at the time of its formation. All our studies have shown the electivity of the tubercle for the vital stain, yet in rabbit 44, where no free stain was available, the tubercles were almost colorless. That failure to stain them vitally was not due to their leucocytic nature or to any refractility to the vital dye was shown by the experiments begun as was rabbit 44, but terminated by a vital dye of another color. In these experiments red tubercles could be grown in blue livers.

Summary.—Within half an hour after the inoculation of tubercle bacilli in vitally stained animals cellular accumulations are found, particularly in the region of the periportal vessels and in the intra-lobular capillaries. The tubercle bacilli are found in clumps free in the centers of the larger cellular masses, but even in the early stages a large number of organisms are found in the bodies of the cells. The polynuclear leucocytes contain relatively few bacteria, but many of the Kupffer cells, even in areas remote from the cellular accumulation, contain organisms. In the later stages the clumps of free bacilli become less conspicuous, and organisms are demonstrable only in the bodies of Kupffer cells. The blue vital stain interferes somewhat with the demonstration of the organisms for it is very intense in the case of cells containing the bacilli. At the end of thirty minutes the plugs in the large vessels are partly composed of a homogeneous blue staining material in which are large numbers of polynuclear leucocytes. Occasional red blood cells and lymphocytes may be found here, but fibrin is not demonstrable. The vessel wall stains vitally, and this is probably the result of an injury brought about by the presence of the organism. The polynuclear leucocytes in the clumps gradually disappear and are replaced by cells which will be described below. At the end of half an hour mitotic figures are relatively frequent in the Kupffer cells, and this process continues through all the stages studied. As a result, Kupffer cells are found at the end of six hours with two or more nuclei, and at the end of twenty-four hours and later stages large brilliantly pigmented multinucleated giant cells lying in the position of the Kupffer cells form one of the most conspicuous elements in the section. The large Kupffer

* We employed dyes whose usefulness in this respect was discovered by Evans and Crowe (Bull. Johns Hopkins Hosp., 1914 (in press)). Diamine fast scarlet to BF is a brilliant red which can be fixed in the tissues in formalin.
cells and giant cells usually contain bacteria, and it is especially around these cells that the formation of the miliary tubercle may best be studied. The giant cell may lie partially or entirely free in the capillary connected only by a streamer to the lining cells. It is stained intensely vitally and, as a rule, it is surrounded by a varying number of cells which may all be stellate or wing-shaped Kupffer cells, containing more or less blue pigment. In this way a miliary tubercle may be found composed entirely of pigmented cells. Some of the Kupffer cells are not pigmented, in which case they are recognizable only by their peculiar form. This involves the separation of the non-pigmented cells from a third type of cell which closely resembles the polyblast. This cell is frequently found in the capillaries, and seems to occur in greater numbers after the six hour stage. Mitotic figures are found in them. They also occur singly, or in nests lying between the Kupffer cells and the liver columns. As a rule, they are not pigmented, but occasionally

The tubercular giant cell noted by Rokitansky and Virchow, and emphasized by Langhans, Wagner, and Schueppel, has caused much discussion, particularly as to whether it is unicellular or multicellular in origin. Weigert, Straus, and Oppenheimer advocate the first theory; Kostenitsch and Wolko Kockel, Miller, and Watanabe consider that it results from a confluence of proliferated fixed tissue cells, and Metchnikoff, Borrel, and Wallgren think that it results from the fusion of white blood cells. Metchnikoff, however, has noted the formation of giant cells in the liver of Spermophilus citillus ("Ziesel") by a budding of the nucleus. Besides the true giant cells, Weigert, Arnold, Kochel, and others describe pseudogiant cells brought about by plugging of small vessels or bile ducts with exudate.

The nuclei of the large Kupffer cells are polymorphous and frequently several almost independent lobes may be found connected by slender strands of nuclear material. It seems probable, therefore, that the nuclei of the giant cells increase by direct division. Rarely pictures occur which suggest that the giant cells may increase in size by fusion with neighboring Kupffer cells. In the center of the larger tubercles deeply pigmented Kupffer cells closely approximated to the giant cell also suggest the possibility of this fusion. Consequently from the evidence that we have we should not deny the ability of more or less separate Kupffer cells to coalesce more perfectly to form the giant cells. But the endothelium of the hepatic capillaries is normally a common syncytial mass, and giant cell formation seems to us to be probably an intense unicellular response rather than an agmination phenomenon. A series of stages based on the unicellular hypothesis can be found in the growth of the young giant cell, although as a whole this question is of subsidiary interest compared with the manner of origin of the tissue.
a few fine granules of blue may be seen in the protoplasm. The cells occur abundantly in the larger tubercles.

Tuberculosis produced in chronically stained animals, in which no trypan blue was present in the circulation at the time of inoculation, resulted in the production of typical non-pigmented miliary tubercles. This is of interest because in chronically stained animals large numbers of deeply stained macrophages are always found in the circulation. These old macrophages are, however, seldom if ever concerned in the young tubercles that arise.

SUMMARY AND CONCLUSION.

In our study of the histogenesis of the miliary tubercle developing inside the liver lobule in animals that have been stained vitally while inoculated with bovine tuberculosis, the controls enable us to recognize the manner in which the vital stain affects the liver. There is therefore no possibility of confusing the effects due to the organism with the effects due to the dye. It is, however, of interest to note that the effects are closely related. The vital stain alone is able to produce gradually some of the same changes that occur with far greater rapidity in experimental tuberculosis. Although in a few hours the Kupffer cells of tuberculous animals begin to react to the disease, in the case of normal animals stained vitally they do not do this until after the third or fourth dose of successive daily injections. After many days, nevertheless, the vital stain alone produces enlargement, proliferation, and separation of Kupffer cells so that these are converted into large free phagocytes which may possess one or several nuclei. These are the gigantic macrophages of chronically stained animals. In all our experiments we have used only acutely stained animals, so that the effects of the dye itself are never sufficient to produce the changes. In fact there is no evidence that the dye accentuates the changes appreciably during the time involved in the experiment. The dye, however, shows us the type of the cells entering into the tuberculous granuloma, for when fed to the body fluids in abundance trypan blue finds its way into all cells capable of receiving it. The vital stain is, as it were, a physiological test for the cells. Whatever the fundamental nature
of the vital stain produced by trypan blue and the benzidine dyes may be, it is important that this reaction does not occur to any appreciable extent with mononuclear blood cells, and that it does occur emphatically in the case of the hepatic endothelium. By means of this vital test, then, the following phenomena occur when suspensions of tubercle bacilli are let into the portal blood stream. The organisms, swept on by the blood stream, finally lodge in the terminal branches of the portal vein, where they plug the vessels and continue to multiply. They injure the vessel wall and cause around them an exudative inflammatory process, and finally lead to the formation of tubercles situated not only in these areas but also within the liver lobule. The injury to the vessel wall is manifested in the early stages by the presence of vitally stained areas in its structure. The bacteria at the end of half an hour are found to be extracellular in clumps in the larger vessels, but already to some extent in the bodies of vitally stained Kupffer cells throughout the liver. Exudative inflammation manifests itself by the presence of a transitory accumulation of polynuclear leucocytes about the bacterial clumps, which may be seen as early as half an hour after the inoculation. They continue to be present in the larger cell clumps of the periportal areas for many days, but they are rapidly replaced by other cells, mononuclear in type, so that within a day the histological appearance of the portal plug has changed radically. The mononuclear cell thus entering most actively into the reaction is endothelial and not hematogenous in origin, the vital stain enabling us to make a clear distinction. This fact, evident in the portal plugs, is decisively shown in the case of tubercles developing within the liver lobule. Such tubercles probably result from the localization of individual organisms within the Kupffer cells, for the initial stages of such a probable cycle have been found by us. They consist of the occurrence of mitoses in certain Kupffer cells where the Ziehl-Nielson method shows a bacillus or several bacilli to have been phagocytized (figure 4). Rapid growth of the infected cell now takes place, and at thirty-six hours the multinucleated giant cell produced is largely separated from the other endothelium of the vessel wall. Many bacilli exist within the protoplasm of these cells (figure 5), which are especially distinguishable
by their intense reaction to the vital stain. They have received trypan blue to such an excess that low power views of liver sections at the thirty-six hour stage show these cells as deep blue spots (figure 8). The origin of the giant cell from the Kupffer cell is evident not only from the above sequence and from the elective stain, but also from the fact that even when fully formed, protoplasmic strands still join it to its mother tissue,—the normal endothelium of the vessel. The strands entangle other cells in their meshes, especially mononuclear blood cells, one of which, of the polyblastic type, has homogeneous protoplasm and is not infrequently encountered in mitosis. These cells are unquestionably of importance in the lesion of tuberculosis. We have seen them abundantly in the capillaries soon after the inoculation and they also occur singly or in nests between the Kupffer cells and liver columns. They are, as a rule, free from the vital dye. They continue to be concerned in the further growth of the tubercle and with the connective tissue cells make the structure of older tubercles relatively complex. On the other hand, little complexity occurs in the structure of the young intralobular masses. The miliary tubercle formed at the end of thirty-six hours is composed of a giant cell, surrounded by epithelioid cells and by blood cells of the above polyblastic type. The giant cell and its so called epithelioid cells are electively stained and are exclusively derived from the hepatic endothelium.

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EXPLANATION OF PLATES.

PLATE 36.

Figs. 1, 2, and 3. Three stages in the mitosis of Kupffer cells from the liver of a rabbit twenty-four hours after inoculation with bovine tubercle bacilli. The large stellate endothelial cells are densely laden with the vital blue granules of trypan blue, 40 c.c. having been given intravenously in two doses. The section is counterstained with aqueous cochineal.

PLATE 37.

Fig. 4. Kupffer cell from the same rabbit as figures 1, 2, and 3. The section is stained by the Ziehl-Nielson method and shows the body of a Kupffer cell in mitosis containing three bacilli. The presence of these might be considered the immediate stimulus for growth of the cell.

Fig. 5. Young endothelial giant cell from the liver of a rabbit thirty-six hours after inoculation with bovine tubercle bacilli, stained as in figure 4 by the Ziehl-Nielson method. The cell is still connected to the vessel wall by protoplasmic processes; within it many bacilli and vital blue granules are found; four nuclei are included in the section; mitosis of any of the nuclei in the giant cell is rarely seen, so that direct division probably occurs.

PLATE 38.

Figs. 6 and 7. Young endothelial giant cells from the liver of the same case as figure 5. The counterstain is aqueous cochineal. The enormous engorgement of these cells with the vital blue is readily seen. As yet no epithelioid cells surround the giant cells. The giant cell of figure 7 is already almost free, but that shown in figure 6 is still part of the endothelial wall, the nuclei of which had increased rapidly, so that a syncytial mass was formed.

PLATE 39.

Fig. 8. Low power view of a thirty-six hour old tubercle, showing the remarkable increase of the vital stain in the tubercle cells as compared with the remaining tissue. The tubercle is still almost purely the central giant cell, though several future epithelioid cells are noticeable.
(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)
(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)
Fig. 8.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)