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Estudio histológico del bazo del conejo durante el aumento de la actividad fagocítica.

El autor ha estudiado la capacidad fagocítica de los esplenocitos del bazo del conejo después de inyectarle corpúsculos sanguíneos lavados de paloma. La hemolisis ordinaria de los corpúsculos de la paloma sigue a la inyección y, casi como un efecto inmediato, en un cierto número de casos siguió una liberación de considerable número de células sanguíneas maduras y no maduras por la médula de los huesos. La sangre hemolizada de la paloma y las células de la médula ósea del conejo, al llegar al bazo transportadas por la sangre, permanecen dentro de los canales sanguíneos cavernosos de la pulpa y comienzan pronto a ser ingeridas por las células esplénicas. Los esplenocitos crecen aumentando su contenido hasta que al cabo de 16 horas adquieren considerable tamaño. Así, por ejemplo, un esplenocito que media $55 \times 23.3 \mu$ presentaba 20 inclusiones celulares en un solo corte de 4μ de espesor. Los esplenocitos que contienen de 5 a 10 células son de menor tamaño. A medida que procede la digestión de las células ingeridas, los esplenocitos disminuyen de tamaño y al cabo de 48 horas muchos de ellos no son mayores que los que se encuentran en el bazo normal. De los productos de la hemolisis de la sangre de la paloma después de la inyección poco queda de un modo permanente en las órganos del cuerpo; la mayor parte se eliminan por los riñones durante las 16 horas. En el bazo los esplenocitos presentan un aumento de substancia férrica, mientras que las células endoteliales no presentan, en su mayor parte, un aumento de tal substancia. En este procedimiento experimental dondequiera que células o fragmentos celulares sirven de estímulo para la fagocitosis, los esplenocitos son los primeros en actuar y continúan actuando como los principales agentes fagocíticos.

HISTOLOGICAL STUDY OF THE SPLEEN OF THE RABBIT UNDER HEIGHTENED PHAGOCYtic ACTIVITY

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ONE PLATE (SIX FIGURES)

The phagocytic reaction in the mammalian spleen has been studied under various experimental conditions and from different points of view. Histologically, the accumulated observations show that the same cell types are not the active phagocytic agents in all cases. Thus in some conditions it is found that fixed cells, such as reticular and endothelial cells, are most active (Heinz, '01; Cary, '15) while in other conditions free cells, such as splenocytes, are the predominant phagocytic cell type (Muir, '02). In other conditions, again, cells of both types take part (Paton and Goodall, '03). These findings are to be correlated with the well-recognized fact that phagocytic cells have a selective action, some types reacting to one stimulus, some to another. With the purpose of examining some of the conditions under which the phagocytic cells of the spleen act, we have studied this organ in one definite experimental procedure, paying special attention to the histological character of the cells involved in the reaction.

The stimulus used has been the injection of washed pigeon corpuscles into the circulation of the rabbit. Rapid destruction of the foreign corpuscles ensues and, as an almost immediate effect, is followed by the release of great numbers of mature and immature blood-cells from the bone-marrow. Both the hemolyzing blood of the pigeon and the bone-marrow cells of the rabbit, being brought by the circulating blood to the spleen, are delayed within the cavernous blood-channels of the pulp, and are there exposed to the phagocytic action of the splenic cells.

EXPERIMENTAL PROCEDURE

The pigeon blood was prepared for injection in the following manner. The pigeon was bled from the jugular veins into 2 per cent sodium citrate in 0.85 per cent sodium chloride solution. Usually 10 to 12 cc. of blood was available, and about 25 cc. of the citrate solution was used to receive the blood. This was centrifuged for fifteen minutes, and the supernatant fluid pipetted off. The corpuscles were next shaken up with 2 cc. of the citrate solution, and then the centrifuge tube filled with 0.95 per cent NaCl solution. The corpuscles were washed twice again, each time first shaking up the corpuscles with a small amount of the citrate solution, and the tube then filled with the 0.95 per cent NaCl solution. Finally, the volume of the washed corpuscles was made up to 10 to 12 cc. with the 0.95 per cent NaCl. Under this treatment there was no visible hemolysis and under the microscope there was seen no clumping of the red blood-cells. Usually 10 cc. of this was injected into a superficial ear vein of the rabbit. The rabbits were taken at varying intervals after injection, from one hour to forty-eight hours after injection. After etherizing the rabbit, the animal was bled through the inferior vena cava and normal saline transfused through the thoracic aorta. After the spleen was partly washed out, as shown by its paler color, the fixing fluid was run in, and attempts were made to distend the spleen as much as possible. The fixing fluid used was a mixture of commercial formalin, made neutral by previously adding magnesium carbonate in sufficient quantity to form a thin layer at the bottom of the stock bottle, and 3 per cent aqueous solution of potassium bichromate. The two fluids were mixed, just before using, in the proportion of one part of neutral formalin to nine parts of the bichromate. Small pieces of the organ were subsequently fixed for a period of twenty-four hours in the same fluid, before washing, dehydrating, etc., preparatory to embedding in paraffin. Hematoxylin and eosin or acid fuchsin were used for routine staining. Tests for the presence of iron were made by the potassium ferrocyanide and hydrochloric acid method.

NORMAL SPLEEN

In the spleen of the normal rabbit can always be found phagocytic cells with inclusions. The detection of these cells is greatly facilitated by spreading apart the constituent tissues of the spleen pulp by intravascular injection of fluids. Although part of the blood content is driven out by this procedure, one can never force out all the blood, and the great advantage is that the resultant preparation is much more easily studied.

The largest of these phagocytic cell types are the free-swimming mononuclear cells, called splenocytes. These vary in size according to the bulk of their contents. The nucleus tends to be rounded or oval in shape, and is usually eccentrically placed. The nuclear membrane is very well defined, and the chromatin is distributed in one or two larger dots and in a network of fine lines. In general the nuclear contents are pale-staining, as compared with the nuclear membrane. The most evident characteristic in the cytoplasm is the presence of inclusions. These inclusions are sometimes single, but are usually multiple. They are most frequently in the form of light yellow spots embedded in the cytoplasm. Indeed, a useful method of detecting the splenocytes is to search over the microscopic field for little clusters of golden-yellow dots. If such a cluster is in a closely compacted mass of cells, diagnosis of the containing cell is very difficult. But if the cell lies free within a pulpar blood space, then the configuration of the cell is readily seen, as shown in figure 1. These contained spots are of varying size, and may not all be of exactly the same color. Sometimes they are closely packed, making a nearly homogeneous mass. In other cells the dots are farther apart, so that the intervening cytoplasm is seen between them. All these appearances are regarded as products of the intracellular digestion of entire erythrocytes, or fragments of them, in the form of hemosiderin. In testing for iron in such cells, these clusters of dots give a strongly positive reaction. Occasionally one sees what appears to be an entire red blood corpuscle still intact within the splenocyte, but final judgment is often difficult. Or again, the inclusions may appear as hemo-

globin-bearing fragments of erythrocytes, as indicated by color comparison with adjacent red blood corpuscles. Not infrequently, too, a splenocyte is seen containing a polymorphonuclear leucocyte.

These polymorphonuclear leucocytes in the rabbit are conspicuous, for in addition to the characteristic shape of nucleus, their numerous fine cytoplasmic granules stain a vivid red color with stains containing eosin. This coloring may lead to some confusion at first in distinguishing them from true eosinophiles. However, the fact that the granules in the latter are larger than in the ordinary polymorphonuclears, and also that their color after eosin staining is more yellowish, helps one to distinguish between them.

SPLEENS OF EXPERIMENTAL ANIMALS

After injection of the pigeon blood, prepared in the manner above described, the corpuscles retain their individuality for but a short time. At the end of one hour they are found to be swollen, rounded, and the nucleus has lost its characteristic staining capacity. Most of the corpuscles are single, but some have begun to agglutinate into little masses. In some elements, the nucleus is still distinguishable by its refractivity. Brown-Sequard, in 1857 (Hunter, '01, p. 120), noted that while dog or rabbit corpuscles injected into the circulation of the domestic fowl could be found at the end of a month, the corpuscles of the fowl injected into the dog or rabbit were no longer recognizable at the end of one hour. Our preparations, in the main, confirm the latter observation.

At this stage, the spleen has grossly a purplish-black color, and its channels are congested with the masses of intermingled rabbit and pigeon corpuscles. It was found that perfusion was even less successful than usual in washing out the blood. A few splenocytes containing inclusions recognizable as entire pigeon corpuscles are seen, but splenocytes with inclusions of amorphous brownish masses are more often to be found, but not in every field, as viewed with the $\frac{1}{12}$ oil-immersion lens. Polymorphonuclears are seen more frequently than in the normal spleen, and

are readily detectable by reason of their red staining granules. No color was yet visible in the urine.

Two hours. At the two-hour period the injected pigeon corpuscles are no longer recognizable as such, having lost their original form and finally become drawn out and subdivided into smaller and smaller globoid and irregular fragments. That the amount of hemoglobin, either free or still in combination with minute portions of the substance of the corpuscles, is overtaking the capacity of the organism is shown by the very evident hemoglobinuria. The cells of the liver show many minute brown granules in their cytoplasm. The number of pseudo-eosinophilic polymorphonuclears has increased, often a dozen being seen in one field. A new and larger type of cell, containing red-staining granules, now appears in small numbers, at most two or three in a field. These are cells with a single eccentrically placed nucleus, which has a very well-defined nuclear membrane. Within the nucleus are one or two larger dots of chromatin, but otherwise the chromatin is very finely divided. The shape of the nucleus usually tends to be spherical, but may have one side flattened or slightly concave. An examination of the bone-marrow shows that similar-appearing cells are there in great numbers. They are both in the blood-channels and in the framework tissue, and most of them, though not all, are packed with the red-staining granules. This comparison leads to the conclusion that the new type of cell now appearing in the spleen has come from the bone-marrow and is of the myelocyte type.

The splenocytes are not greatly different in number or size from before. Some are conspicuous by their multiple brownish-colored inclusions. One of these cells measured $20.5 \mu \times 12.3 \mu$, which is no larger than splenocytes, containing several inclusions, from the normal spleen. Occasionally a splenocyte is seen containing a pseudo-eosinophile, in addition to remains of red blood corpuscles.

Three hours. At three hours after injection, the pigeon corpuscles have been reduced to brown-colored masses and clumps of fine particles. Even more intense hemoglobinuria is found than at the preceding stage. Polymorphonuclears and myelo-

cytes are present in about the same numbers as before. The splenocytes contain brown masses derived from the ingested particles of blood. These may exist as a single large mass in a cell or as many small ones. The average size of the splenocytes has not increased to any extent.

Four and one-half hours. Fragments of the injected pigeon corpuscles are still abundant, intermingled with the rabbit corpuscles. The polymorphonuclears have increased in number and myelocytes are frequent. The splenocytes may contain a single brownish mass, or more frequently many inclusions, usually brownish globules and often leucocytes. One cell was seen containing seven polymorphonuclears, as well as many fine brown dots. The average size of five splenocytes was $16.7 \times 10.8 \mu$, with nucleus $6.8 \times 5.9 \mu$. Measurements of five myelocytes showed average size of cell-body of $10 \times 8.7 \mu$, with nucleus $7 \times 5 \mu$. The average size of the pseudo-eosinophilic polymorphonuclears, as seen in sections, is $6.5 \times 6 \mu$.

Six hours. After six hours, the appearance of the sections is very striking from the presence of numerous myelocytes, with red-staining granules. What appears to be mitotic division in these cells is seen not infrequently (fig. 2, *My*). Whether this may be considered a normal mitotic division has been questioned.

The polymorphonuclears (fig. 3) are even more numerous than the myelocytes, but the latter on account of their larger individual size form the most conspicuous elements in many fields. Splenocytes are scattered here and there and have inclusions similar to those in the preceding stage. There are occasional extracellular masses of yellowish hemosiderin. Several megacytes were seen, and these are apparently cells which have remained in the spleen from fetal life, for in rodents' spleens such cells are found not infrequently. One of these is shown in figure 3 (M), and its size is useful for comparison with the size of the splenocytes in their several stages of phagocytic activity. The hemoglobinuria still persists. Within the endothelial cells of the blood-channels very fine yellowish granules could be seen. Tests for the presence of iron showed this to be present within these cells and also very strikingly within the splenocytes.

Eight and one-half hours. The appearance at this stage is similar to the preceding. There is an abundance of myelocytes and of pseudo-eosinophiles. The splenocytes usually contain numerous small inclusions of hemosiderin and are not very conspicuous.

Twelve hours. The splenocytes are now beginning to show inclusions of nucleated cells, in addition to the blood pigment derived from the pigeon red blood corpuscles. This is the last stage at which hemoglobinuria was noted, and apparently the pigeon blood has for the most part been disposed of. The phagocytosis of the bone-marrow cells by the splenocytes is now commencing, and shows most strikingly at the next stage.

Sixteen hours. At this period, one can readily discern the splenocytes with a low power of the microscope, by reason of their increased size. In figure 4 is shown a characteristic field. Every splenocyte contains a number of nucleated cells, both polymorphonuclears and myelocytes with their granules still staining characteristically. An average size is $25 \times 20 \mu$, in contrast to their previous size of $16 \times 10 \mu$. A large one, shown in figure 5, measures $55 \times 23.4 \mu$, and in single 4μ section, twenty cells can be counted within its cytoplasm. This period evidently shows the splenocytes at the height of their phagocytic activity toward the animal's own bone-marrow cells. There are scattered extracellular masses of hemosiderin. The polymorphonuclears are still abundant, but the myelocytes are apparently not so numerous, and this is probably due to the activity of the splenocytes. At this period there was no discernible hemoglobinuria. Tests for iron showed this to be present in many endothelial cells, but in much greater amounts within splenocytes.

Twenty-one hours. The splenocytes are now smaller in size, and accordingly less conspicuous than at the preceding period. Their diminution in size is to be associated with the shrinkage of their cellular inclusions. While in some of the splenocytes, the ingested cells show both nuclei and granules stained characteristically, in others, only the nuclei of the ingested cells remain recognizable, and apparently the red-staining substance has been altered, probably by digestive ferments within the cell. Some

splenocytes also contain small yellowish or light brown inclusions derived from the original pigeon blood. Occasionally a splenocyte is seen containing one single larger rounded mass of hemosiderin. While polymorphonuclears are still scattered about, no distinct myelocytes were found.

Forty-eight hours. The splenocytes are again easily detectable in the microscopic field, because of the deep staining of the remains of the cells which they had ingested. These cellular inclusions, for the most part, no longer resemble the original cells. The nuclei show as shrunken round little masses, which stain intensely with the hematoxylin, and no remains of their cytoplasmic granules are seen. The size of the splenocytes has much diminished, and has returned nearly to the size seen at the early stages. A characteristic one, with a dozen little dark-stained dots within it, measured $18 \times 15 \mu$. Others, in which the digestion of the cells has not advanced so far, are of larger size. In some cells, in addition to the shrunken remains of the nucleated cells, are seen yellowish granules, derived from the red blood corpuscles of the pigeon. The polymorphonuclears are few in number, and no myelocytes remain.

DISCUSSION OF OBSERVATIONS

In considering the results of these experiments there are several variables, which have to be taken into account. First, in the washing out of the spleen, the perfusion is more successful in removing the contents of the vascular channels in some cases than in others. However, as mentioned before, the result is never complete washing-out, and by studying various areas in the sections one can reach definite conclusions as to the relative number of the different types of cells present. A second and even more important point is the difference in amount of reaction which the bone-marrow gives. In some animals apparently great numbers of cells are released and in others relatively few. This may depend upon the age of the rabbit, but many other factors must be considered before a solution can be arrived at.

The outstanding features of the experiments are: 1) the definite cycle which can be followed in the phagocytic activity of the splenocytes, and, 2) the bone-marrow crisis occurring as a reaction to the hemolysis of the pigeon corpuscles.

In following the phagocytic history of the splenocytes, one notes that the hemolysis of the pigeon blood is so rapid that few of these cells are taken up intact. It is true that some are taken up whole, but as dissolution proceeds, it is more and more the fragments which are ingested by the splenocytes. At the one-hour stage, the reaction has already begun, some of the inclusions being recognizable as entire red blood-cells, others as amorphous masses derived from the pigeon corpuscles. However, the splenocytes are not greatly enlarged, and even by the end of six hours, after an interval of several hours during which the splenocytes have been surrounded by the disintegrating blood-cells, their size is not conspicuously increased. Either the output from the cells keeps pace with the intake or the phagocytic process requires a number of hours for its completion. That time is a necessary factor in the engulfment stage of phagocytosis is also indicated in the action of the splenocytes toward the bone-marrow cells. These first appear in the spleen at the two-hour stage, and are abundant by the six-hour stage, but it is not until the twelve-hour stage that any conspicuous number of these cells are seen within the splenocytes. Thereafter the engulfing process continues rapidly, and at sixteen hours, practically all splenocytes are laden with these cells.

As the result of the ingestion of myelocytes and polymorphonuclear cells, the size of the splenocytes greatly increases. An average size is $25 \times 20 \mu$, but many are larger than this, and the example shown in figure 5 measures $55 \times 23.4 \mu$. In this cell at least twenty bone-marrow cells can be counted within its cytoplasm in a single 4μ section, so that one can judge that its total capacity must be several times this number.

After the stage of engulfment comes the digestion of the cells. First the pseudo-eosinophilic granules lose their characteristic staining capacity, although the nuclei still show their original form. Gradually, however, the nuclei begin to shrink, and

apparently the polymorphous nuclei separate into small pieces. These shrunken nuclear remnants stain more deeply than do the original nuclei. The twenty-one-hour material shows splenocytes containing some cells which still show red-stained granules, and other cells in which the granules are no longer apparent.

At the forty-eight-hour stage most of the inclusions have been reduced to the condition of fine dark-staining granules, while others (fig. 6) may still retain the original form of nucleus. Those splenocytes (fig. 6) which contain cells which have been altered but little, are usually larger than those in which the digestion of the cells has advanced farther. Indeed, many of the splenocytes are now but little larger than some found in the normal spleen. They are, however, quite conspicuous, on account of the intense staining of the nuclear remains within their cytoplasm. The digestion of the cells by the enzymes of the splenocytes is evidently a process which requires considerable time. At the twenty-one-hour stage no free myelocytes were found, and relatively few polymorphonuclears, and it appears that the splenocytes even at the sixteen-hour stage, were already well filled. So it would seem that the cells ingested at sixteen to twenty-one hours had not been completely digested by the forty-eight-hour stage.

These experiments indicate that the splenocytes can ingest material from a very small size to cells $16 \times 10 \mu$ in dimension. They also readily take up the ultramicroscopic aggregates of trypan blue. So it is apparent that they have a very wide phagocytic capacity as regards size of object to be ingested.

Viewing this in relation to their activity toward effete red blood corpuscles of their own circulation, one must conclude that they are capable of taking up either entire red blood-cells or fragments of them. In a recent paper, Rous and Robertson (17) have found that phagocytosis of entire red cells is frequent in normal dogs, rats, and guinea-pigs, slight in man, the rhesus monkey, and many rabbits. This phenomenon is practically absent in cats and in some dogs and some rabbits. In these latter they find that fragmentation of the red cells occurs, and they question how the fragments are disposed of. Judging by the

range of activity of the splenocytes, one must conclude that the splenocytes are just as capable of ingesting schizocytes as of taking up the entire red blood-cells and that phagocytosis must still be regarded as a sufficient general explanation of normal blood destruction.

Cary ('15) studied the method of disposal of bovine corpuscles when injected into the blood stream of the rabbit. His method of study was by applying the reaction for iron. He found the injected corpuscles to be taken up by hemophages, which he indicates are fixed cells. In contrast to these results, we have found free-swimming cells to be the most active phagocytic agents in the spleen. Muir ('02), studying the rabbit's spleen in infections, found non-granular free cells containing as many as twenty or more red corpuscles and a half-dozen leucocytes in various stages of disintegration. This is a reaction comparable in many ways to that which we have been following.

Muir also observed myelocytes in the splenic pulp, in infectious conditions, especially when severe. The greatest numbers he found in cases of variola. Once or twice he observed them undergoing mitosis. The bone-marrow crisis is evidently the result of the action of the toxic material set free in the hemolysis of the pigeon blood. That it begins between one and two hours after the injection is indicated by the myelocytes being seen in the spleen at the two-hour stage. This time coincides with that at which the crisis of white cells takes place after acute hemorrhage, and it is to be noted how promptly the spleen reflects the bone-marrow crises. By four or five hours after injection of the foreign blood, great numbers of the bone-marrow cells have been caught within the spleen. These are detained within the cavernous blood-spaces, and finally ingested by the splenocytes.

SUMMARY

The cycle of changes associated with the phagocytic activity of the splenocytes of the rabbit has here been followed.

When washed pigeon corpuscles are injected intravenously, they are rapidly hemolyzed. The hemolysis of the pigeon blood results in the liberation of great numbers of bone-marrow cells, mature and immature. These are caught within the spleen, and quickly begin to be ingested by the splenocytes. The splenocytes grow with their increased contents, until at the sixteen-hour stage they reach a very large size. As many as twenty cells are visible in a $4\ \mu$ section of a splenocyte, measuring $55 \times 23.4\ \mu$. As digestion proceeds, the splenocytes become smaller, and at the forty-eight-hour stage they are much reduced in size, some not much larger than normal.

Of the products of hemolysis of the pigeon blood after a single injection, little remains within the organs of the body and most is excreted through the kidneys within sixteen hours. In the spleen, the splenocytes have an increased amount of iron-containing substances, while the endothelial cells show, for the most part, very little. The comparatively small results seen within the phagocytic cells follow from the rapid reduction of the foreign blood corpuscles to particles of a very small size, and from the short time in which these fragments remain within the blood stream.

In this special experimental procedure, where cells and cell fragments are the stimulus to phagocytosis, the splenocytes are the first to act, and they continue to act as the main phagocytic agents.

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PLATE 1

EXPLANATION OF FIGURES

The outlines were first drawn with the aid of a camera lucida at a magnification of 770 diameters. In the reproducing of the drawings the illustrations were reduced to 575 diameters. The sections from which the drawings were made were 4μ thick and stained in hematoxylin and eosin.

1 Normal spleen of rabbit, showing several splenocytes with inclusions. *Sp*₁, splenocyte with single inclusion, colored with blood pigment. *Sp*₂, splenocyte with several vacuole-like areas in its cytoplasm. *Sp*₃, splenocyte with inclusions of polymorphonuclears.

2 Spleen of rabbit, six hours after injection of pigeon corpuscles, showing myelocytes in the vascular channels. *Sp*, splenocyte with inclusion colored with blood pigment. *B*, extracellular mass of blood pigment. *My*₁, myelocyte showing stage of mitotic division, with chromosome-like masses of chromatin. *My*₂, myelocytes with pseudo-eosinophilic granules.

3 Spleen of rabbit, six hours after injection of pigeon corpuscles, showing myelocytes (*My*), pseudo-eosinophilic polymorphonuclear leucocytes (*P*), megacyte (*M*), splenocyte (*Sp*), and blood pigment, both intracellular and extracellular.

4 Spleen of rabbit, sixteen hours after injection of pigeon corpuscles, showing three splenocytes with cellular inclusions. *N.Sp.*, nucleus of splenocytes.

5 Spleen of same rabbit shown in figure 4. Shows a splenocyte greatly enlarged by reason of its numerous cell inclusions. *N.Sp.*, nucleus of splenocyte; *R*, reticulo-endothelial cells.

6 Spleen of rabbit, forty-eight hours after injection of pigeon corpuscles, showing two splenocytes with inclusions. The splenocytes (*Sp*) are now becoming smaller, as the digestion of the phagocytosed cells approaches the end of the process.