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NORMAL AND PATHOLOGICAL FRAGMENTATION OF RED  
BLOOD CELLS; THE PHAGOCYTOSIS OF THESE  
FRAGMENTS BY DESQUAMATED ENDOTHEL-  
IAL CELLS OF THE BLOOD STREAM; THE  
CORRELATION OF THE PEROXIDASE  
REACTION WITH PHAGOCYTOSIS  
IN MONONUCLEAR CELLS.

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In 1917, Rous and Robertson (1, 2) demonstrated that there is a constant fragmentation of red cells in the circulating blood of the normal animal. They drew blood directly from the hearts of mammals into gelatin-Locke-citrate and obtained microcytes and poikilocytes by differential centrifugation. They observed that fragmentation was increased in anemia and also by such mechanical factors as pressure and heat. The process had been seen by many observers; for example, by Ehrlich (3), Arnold (4), Schwalbe (5, 6), Maximow (7), Weidenreich (8), and others, with varying interpretations, but it was Rous and Robertson who determined that it is a constant and, in their judgment, the major factor in the destruction of red cells.

We have been able to confirm the concept that there is a constant fragmentation of red cells in the circulation of the normal rabbit by the study of films of fresh blood, and have found that the fate of these fragments is to be taken up by the so called clasmatocytes or wandering endothelial phagocytes. We shall use the three terms: clasmatocyte, wandering endothelial phagocyte, and macrophage as synonymous and distinct from monocyte. Clasmatocytes with fragments of red cells are to be found in the circulating blood, in the spleen, and in the connective tissues, and we think that they can be discriminated, when studied with the supravital technique, from eosinophilic leucocytes, with which they have been confused in the literature. Thus,

clasmatocytes not only take in and destroy whole red cells, as can be demonstrated in the spleen and bone marrow of any normal rabbit, but they also dispose of the results of the fragmentation of red cells which is constantly going on in the blood stream. With any increase in fragmentation there is an increase in the number of clasmatocytes containing these fragments. If it should prove correct that the clasmatocyte is a derivative of endothelium, then it will be interesting that this strain of cells should carry the major part of the work of breaking down and conserving hemoglobin, whether by phagocytosis of whole red cells, or of their fragments, inasmuch as it is endothelium which has the power of building up hemoglobin (Doan *et al.*, 9).

To study the process of fragmentation, we have made preparations of rabbit blood on films of dilute neutral red, sealed them as rapidly as possible to limit crenation, and watched them in a warm box. Because the process is markedly increased by a rise in temperature, as was seen by Weidenreich and by Rous and Robertson, we have taken pains to note that the phenomenon can be found immediately after the preparation is made, before the film, taken at room temperature, has had time even to come to the temperature of the warm box, 37°C. Indeed, an increase in fragmentation is so constant with a rise in temperature above 37° that it serves as a check on the accuracy of the regulator in the warm box.

In watching fragmentation, we have found that a red cell puts out a long process and thereby becomes the so called poikilocyte. At first, the process does not move, then it begins to vibrate slowly, then faster and faster. This vibration marks the beginning of fragmentation. As the process vibrates, it gradually becomes thinner and thinner at some point, and then separates. If the thinned out place is at the base of the long process, the fragment becomes a rod, often looking like an irregular but yellow bacillus; if near the tip, it becomes a round or oval body. We have seen every variation in size and shape of these processes, up to an actual division of a red cell into two equal parts. Sometimes two or more processes form on a red cell at the same time. When the blood of patients with pernicious anemia is studied with reference to fragmentation, the poikilocytes show the same marked vibration of the processes as in secondary anemia, but they may not separate in supravitral preparations even for 24 hours.

The blood shows many bizarre shaped fragments, nevertheless. It is thus possible that the large number of poikilocytes present in this disease may be associated with a delayed fragmentation. We interpret a poikilocyte as the first stage in the process of fragmentation, and the microcyte, in many instances at least, as a red cell reduced in size after some of its cytoplasm has broken off.

*Phagocytosis of Fragments of Red Cells by Desquamated Endothelial Cells in the Blood.*

The anemia which always occurs in rabbits infected with tuberculosis has been followed, and it has been found that there is an increase in fragmentation as the number of red cells and hemoglobin diminish. Fragmentation in connection with anemia was first seen by Ehrlich and studied by Rôus and Robertson. In the present experiments supravital differential counts of the peripheral blood were made every other day. In the accompanying paper in this journal (10), it was shown that there is a constant desquamation of endothelial cells into the blood stream in normal rabbits and that the desquamation is markedly increased at certain stages in tuberculosis. In preparations of living blood, we have found phagocytosis of the fragments of red cells by free endothelial cells. These studies have afforded an especially good opportunity to compare the clasmatocytes with included fragments of red cells with true eosinophils. As is well known, the number of true eosinophilic leucocytes is low in rabbit blood, averaging about 2 to 3 per cent in the normal animal. In our experience, eosinophilic leucocytes disappear altogether from the blood stream in rabbits in from 24 to 72 hours after the intravenous injection of tubercle bacilli, and they almost never appear again in the blood unless the animal survives and the blood cells otherwise return to normal. After about 3 or 4 weeks, when the infected rabbits show an anemia having around 3,000,000 red cells and a hemoglobin of about 35 per cent, a few of the endothelial phagocytes in the blood appear loaded with fragments of red cells. Most of the cells are well filled with the fragments, but some have been found containing only one or two fragments. In the supravital technique it is easy to distinguish these cells from true eosinophilic leucocytes, first by the

character of the granules, and second by the different type of motility of the two cells. The eosinophilic granules of the rabbit's cells are large and oval in shape. When unstained, they are slightly greenish and highly refractive; in supravital neutral red, they react toward the orange shade shown by the dye in dilute alkaline solution. In the true eosinophilic leucocytes, there are also a few tiny red bodies in the scarlet color of the acid reaction of the dye, which we interpret as vacuoles. They are round and vary in size. The fragments of red cells, on the other hand, have exactly the color of the hemoglobin in the surrounding red cells when they have just been taken into the clasmatocyte. After they have been engulfed for a time, they begin to show a pink color in neutral red, which we interpret as the color of the fluid of digestion which the cell secretes around them. The fragments of red cells vary more in size and shape and are less refractive than eosinophilic granules. As the fragments are increasingly affected within the cell through the breaking down of the hemoglobin, they shrink and stain more and more deeply with the dye. At no stage are they like eosinophilic granules in the fresh, unstained films, or in their reaction to supravital staining.

In type of motility also the two cells are different. The true eosinophilic leucocyte has typical ameboid motion, with streaming of the granules as the cell moves rapidly across the field. The clasmatocyte, on the other hand, moves so sluggishly that one has to watch it for some minutes to see that it is changing at all.

Weidenreich (11, 12) proposed the theory that the granules in eosinophilic leucocytes are fragments of red cells. He found large numbers of eosinophilic cells in sections of hemal glands. They were within the blood spaces of the gland and in these same spaces there were large masses of fragments of red cells. He noted that the fragments outside the cells had an exactly similar reaction to Orange G and to eosin as the granules within the cells. Weidenreich discriminated between eosinophilic leucocytes and clasmatocytes with fragments of red cells by the difference in size and in the nuclei of the two cells, but he concluded that the granules were the same in both cells and that they were fragments of red cells. We think, on the contrary, that while the two types of so called granules cannot always be discriminated in fixed tissues, they can be distinguished in the living state,

both when unstained and when colored with neutral red. Concerning the presence or absence of iron in eosinophilic granules there are discrepancies in the literature, but the matter has not been considered from the standpoint of discriminating true eosinophilic leucocytes from clasmatocytes with fragments of red cells. Barker (13) found that the eosinophilic granules in leucocytes of patients with malaria and in other conditions in which the eosinophils were increased gave a reaction for masked iron. Neumann (14) has recently reported that the eosinophilic granules in the leucocytes of the horse are free from iron. Some method of discriminating these two types of cells, containing granules reacting alike to eosin, in sections of pathological material, is desirable.

In the present study the tuberculous rabbits were killed at the stage in which it was expected that a maximum grade of the disease in the lungs would be found, as indicated by the increase in modified monocytes in the peripheral blood, shown by Cunningham, Sabin, Sugiyama, and Kindwall (15). This stage coincides with a period of marked and increasing anemia. Supravital studies of scrapings of the fresh lungs, spleen, and bone marrow were made. In every instance increased numbers of clasmatocytes loaded with fragments of red cells were found in the tubercular tissue of the lungs; also they were always present in the spleen and bone marrow. Thus, it seems certain that, while marked fragmentation occurs as an evidence of increased disintegration of red blood cells in many pathological states, phagocytosis and not laking or ultimate extracellular destruction is, to say the least, a frequent fate of these fragments, as well as of the senile whole red blood cells.

One of our rabbits (R 11) showed considerable resistance to the tuberculous infection, for it was inoculated with the bacilli on November 16, 1925, and survived to March 6, 1926, when it was killed. The animal was in good condition at this time. It weighed 2020 gm. originally; the lowest weight was 1765 gm. on December 18, 1925, but the weight had returned to 1985 gm. on February 17, 1926. The red cells were originally 5,592,000, with a hemoglobin of 74 per cent (Newcomer); the greatest anemia was on December 8, 1925, with a count of 2,800,000 red cells and a hemoglobin of 44 per cent; but the red cells had returned to 6,570,000, with hemoglobin of 77 per cent the day the animal was killed. The tissues of this animal were studied for clasmatocytes with fragments of red cells; in the spleen there was the usual number of clasmatocytes with whole red cells,

but none was found with small fragments. The right lung was badly infected with large tubercular regions, and many small cavities, and in the septa of this lung many clasmatocytes were found loaded with whole red cells or with very large fragments, but none with the small fragments that could be confused with eosinophilic granules. Thus, large numbers of clasmatocytes loaded with fragments of red cells may be associated with an anemia. The left lung was normal in appearance in the gross, but showed some epithelioid cells in the septa.

*Reactions of the Two Strains of Mononuclear Cells of the Blood to the Peroxidase Stain.*

A method of studying blood, by which it is possible to discriminate desquamated endothelial cells from monocytes, and a condition such as tuberculosis in rabbits, in which both strains of cells are markedly increased, offer favorable material for the study of differential oxidase reactions. Concerning the oxidase reaction of mononuclear cells, there are many discrepancies in the literature.

The monocytes of human blood were described as positive to the oxidase reaction by Naegeli (16, page 144). Reschad and Schilling-Torgau (17), and Schlenner (18), using another technique, reported them negative. Reschad and Schilling-Torgau studied the oxidase reaction in monocytes in a case of monocytic leucemia in which the discrimination of the cells in fixed films was accomplished by means of the azurophilic granules, as well as by a marked development of a centrosphere around a centrosome. Schlenner studied the monocytes which are present in increased percentage in cases of malaria. In analyzing the findings of Schilling and of Schlenner, Katsunuma (19) found that the human monocytes are regularly negative with their technique, while they are positive with the original technique of Schultz. It is therefore essential to state the exact technique in every study of an oxidase reaction. Recently, McJunkin (20) has questioned the presence of any monocytes in the blood of the rabbit on the score that the so called monocytes of the rabbit are negative to the oxidase test, while the monocytes of human blood are positive. It has been shown by Katsunuma, whose recent monograph on both stabile and labile reactions in general is most comprehensive, that the monocytes of the blood of rabbits, guinea pigs, apes, dogs, rats, and mice can be regarded as negative in a general way to the stabile oxidase test, and yet they give a very weak reaction of a diffuse blue color without definite, colored granules, a reaction which can be brought out with faint illumination, in contrast with the entirely negative, stabile oxidase reaction of lymphocytes. Thus, he concludes that the oxidase substances are perhaps less ripe in the monocytes of the animals just mentioned than in human monocytes, so that they are easily overlooked. Moreover, he showed that in human blood there are always some monocyte-like cells that are negative.

Our findings agree with those of Katsunuma and provide a further analysis of the reaction in mononuclear cells.

We have used the peroxidase technique of Sato and Sekiya, as given by Sato and Yoshimatsu (21), which involves a mordant of copper sulfate, benzidine and hydrogen peroxide, and a counterstain of safranine. The method gives clear-cut results, which are constant when the blood is stained the day it is drawn; and the reagents are stable. The counterstain of safranine gives the best differentiation we have found for identification of the type of cell involved. As is well known, the difficulty in interpreting the type of cell taking an oxidase reaction is great, because the especial characteristics by which the cells have been discriminated are not brought out. For every preparation with the peroxidase reaction we have made a differential count of consecutive drops of blood with the supravitral technique and with Wright's stain, and compared the three different techniques. In the stage of tuberculosis already described as being studied, there is always a marked depression of the activity of the bone marrow, so that besides the anemia the leucocytes with fine granules are diminished, at times to as low as 12 per cent. The less massive oxidase reaction in the rabbit leucocytes makes their characteristic nuclei readily visible. Normal rabbit blood was taken for the reaction of the eosinophilic leucocytes and it was found that they show a massive blue peroxidase granulation either general or in a rim at the periphery of the cell, so that they are distinctive and markedly positive. The basophilic cells, on the contrary, which are not uncommonly as high as 10 per cent in the normal rabbit and are not reduced in tuberculosis, show bright red granules, rather than the blue granules of the other myeloid cells with the Sato and Sekiya method. At least there are cells with red granules that check in the same percentage and physical appearance with the basophils of the differential counts in the other two methods. An occasional cell in human blood shows the same reaction. The lymphocytes are, of course, uniformly negative, as in all stable oxidase and peroxidase stains.

With the desquamated endothelial cells, there is the most interesting and varied reaction to the peroxidase stain. It is known that endothelium itself is negative. Katsunuma, however, has demonstrated that the Kupffer cells will yield a positive reaction when they

have phagocytized positive material. His experiment was as follows: he injected the positive eosinophilic granules freed from the leucocytes of the horse into the mesenteric vein of rabbits and found that the Kupffer cells, as they phagocytized this debris, became positive. In the rabbit the desquamated endothelial cells of the blood are negative or positive, according to their physiological state. As was shown by the supravital technique (10) in the accompanying paper, some of the endothelial cells in the blood do not stain in neutral red, others stain partially or completely; some are obviously degenerating, while others are motile and contain substances that can be identified as ingested debris, indicating that the cells are functioning. In the Sato and Sekiya technique, we discriminate the endothelial cells from monocytes and from lymphocytes in part by their thin, filmy cytoplasm and characteristic nuclei, and in part by their shape. We divide (10) the desquamated endothelial cells into four groups; small cells, the size of the small lymphocyte, cells of intermediate size, up to the size of the largest monocyte, very long cells, and giant cells. In the blood of tubercular rabbits there are small cells, the size of a small or intermediate lymphocyte, often 3 to 4 per cent of them, which have positive blue granules in the peroxidase films. This is confirmation that their discrimination from lymphocytes by the supravital technique is justified, since no lymphocytes have been shown with a stabile oxidase reaction.

In the studies made of the rhythm of the leucocytes from human blood, by Sabin, Cunningham, Doan, and Kindwall (22), it was found that there was a discrepancy in the counts made by the vital technique and on fixed films, which was invariably in one direction, namely, toward an increase in the cells estimated as lymphocytes, with a consequent decrease in leucocytes in fixed films, as compared with the number found by the supravital technique. The average discrepancy was about 5 per cent, but it was as high as 10 per cent in certain individual counts. The same discrepancy appears with rabbit blood. Some of the counts made by the two methods agree closely, but again there occur discrepancies in the direction of an excess of lymphocytes in the fixed films. We have now come to the conclusion that this discrepancy is due to small and medium sized clasmatocytes, which have heretofore been overlooked by us in supravital films and have

been included as lymphocytes in fixed films. We think that the free nuclei of degenerating, small endothelial cells, and perhaps of other cells, which are indistinguishable from debris in fresh films, are often included as small lymphocytes in fixed films. In the accompanying communication (10) evidence has been given to show that the majority of smudges of fixed films are of nuclear origin. To approximate accuracy in fixed films with reference to small lymphocytes, it is essential to have the original dilution of the methylene blue-azur in methyl alcohol adjusted to the particular blood to be stained, as a too intense basophilic reaction obscures the thin rim of cytoplasm in the smallest lymphocytes.

The endothelial cells of medium size, the long types, and the giant forms are negative, positive, or contain a mixture of the positive blue

TABLE I.

	No. of cells.	Total No. of endothelium.	Endothelium positive to peroxidase.	Endothelium negative to peroxidase.	Total No. of monocytes.	Monocytes positive to peroxidase.	Monocytes negative to peroxidase.
Rabbit blood (tubercular).	3200	184 5.7%	54 29%	130 71%	534 16.7%	2 0.37%	532 99.63%
Human blood (normal).	3000	64 2%	43 67.2%	21 32.8%	177 5.9%	169 95.5%	8 4.5%

granules and the red granules similar to those of the basophilic leucocytes. These cells may have one massive blue granule, two or three tiny granules, many tiny granules, or granules massively clumped or scattered throughout the cytoplasm of the cell. The clasmato-cytes which are filled with fragments of red cells are negative to the peroxidase reaction in contrast to the decidedly positive reaction of the true eosinophilic leucocytes. This finding was checked in tissues where the number of these cells was known to be great from supravital studies, as well as in the peripheral blood. The percentages of the endothelial cells positive and negative to the peroxidase reaction, as compared with monocytes, are shown in Table I.

The conclusion has been reached that the desquamated endothelial cells found in the circulating blood are in part degenerating types, but in part actively functioning cells that are phagocytic in the blood

stream. Under pathological conditions these clasmatoocytes have been reported as containing whole leucocytes; they probably also phagocytize fragments of leucocytes containing neutrophilic granules, which are frequently found in fresh films of blood.

It can be said of the monocytes that almost all of this type of cell in human blood are positive to the technique of Sato and Sekiya; but, as shown in the table, an occasional one is negative. In the estimation of this negative reaction, only cells of large size, with an excentric and indented nucleus, the typical transitional in the terminology of Ehrlich, were included as monocytes. That there is an occasional negative, large mononuclear cell in human blood agrees with the findings of Katsunuma. Moreover, with the positive monocytes of human blood, there is the greatest possible variation in the number of the blue granules, from a reaction of numerous fine granules, scattered throughout the cytoplasm, down to one positive granule. With the monocytes of the blood of rabbits the reverse condition is true, because the vast majority of their monocytes lack the granules and only an occasional cell is positive. Here also the greatest care must be taken in the study to exclude two other types of cells, namely, large lymphocytes and clasmatoocytes of appropriate size. Only a cell quite clearly of the type of the transitional cell of Ehrlich was considered, a large cell with dense cytoplasm and with an excentric and indented nucleus.

We therefore conclude that most of the monocytes of human blood have positive peroxidase granules with great variation in the number of these granules, but that a few monocytes lack them entirely; while, in contrast, most of the monocytes of rabbit blood lack the positive blue granules, but a few contain them. Thus, the difference in monocytes in various species seems to be more one of degree than of kind, and therefore not a basis for a classification, but rather an indication of a difference in functional activity. The epithelioid cells of the tuberculous lesions in the rabbit have been found negative, as in the case of the monocytes from which we believe they are derived, except in rare instances, in which a leucocyte containing positive granules had clearly been phagocytized. The monocytes, epithelioid cells, and giant cells from human tubercular lymph glands have<sup>1</sup> been found

<sup>1</sup> Through the courtesy and cooperation of Dr. John Hanford, of the Surgical Staff of the Presbyterian Hospital, we have had access to fresh human tubercular lymph glands (cervical) removed at operation.

to be positive to the peroxidase reaction, having fine blue granules comparable to the reaction in the 15 per cent of positive monocytes found in the peripheral blood of the same patient the same day. Thus, the essential identity of the monocytes of rabbit and human peripheral blood and their respective relationship to the specific cellular reaction in tuberculosis would seem to be entirely compatible with the peroxidase reaction occurring in these cells. Clasmatocytes filled with fragments of red blood cells and negative to the peroxidase reaction were conspicuously present in these human lymph gland preparations.

#### CONCLUSIONS.

1. There is constantly some breaking down of red cells in the circulation by fragmentation.
2. The fragments of red cells, as well as whole red cells, are phagocytized and destroyed by clasmatocytes or endothelial phagocytes.
3. When there is an increase in fragmentation in abnormal or pathological states, desquamated endothelial cells of the blood stream, as well as the clasmatocytes of the tissues, increase proportionately and take in these fragments. These cells are to be distinguished from eosinophilic leucocytes by the nature of their granules, by the type of motility of the cells, and by a negative peroxidase test.
4. The desquamated endothelial cells, clasmatocytes, in the circulating blood are positive to the peroxidase test only when they have taken in positive material.
5. The monocytes show marked variations of the oxidase reaction in different species and to different techniques. With the Sato and Sekiya technique most monocytes of human blood are positive, while most of them in rabbit blood are negative, but both positive and negative reactions are found in both human and rabbit blood.

#### BIBLIOGRAPHY.

1. Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651; *Studies from The Rockefeller Institute for Medical Research*, 1917, xxvii, 163.
2. Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665; *Studies from The Rockefeller Institute for Medical Research*, 1917, xxvii, 177.
3. Ehrlich, P., *Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes*, Berlin, 1891, pt. 1, 99.

4. Arnold, J., *Virchows Arch. path. Anat.*, 1899, clv, 165.
5. Schwalbe, E., *Ergebn. allg. Path. u. path. Anat.*, 1902, viii, 150.
6. Schwalbe, E., *Beitr. path. Anat. u. allg. Path.*, 1905, suppl. 7, 52 (Festschrift für Dr. Julius Arnold).
7. Maximow, A., *Arch. Anat. u. Physiol., Anat. Abt.*, 1899, 33.
8. Weidenreich, F., *Anat. Anz.*, 1903-04, xxiv, 186.
9. Doan, C. A., Cunningham, R. S., and Sabin, F. R., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 163.
10. Sabin, F. R., and Doan, C. A., *J. Exp. Med.*, 1926, xliii, 823.
11. Weidenreich, F., *Anat. Anz.*, 1901, xx, 188.
12. Weidenreich, F., *Anat. Anz.*, 1901-02, xx, 193.     •
13. Barker, L. F., *Bull. Johns Hopkins Hosp.*, 1894, v, 93.
14. Neumann, A., *Z. wissenschaft. Biol., Abt. B*, 1925-26, iii, 46.
15. Cunningham, R. S., Sabin, F. R., Sugiyama, S., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 231.
16. Naegeli, O., *Blutkrankheiten und Blutdiagnostik*, Berlin, 4th edition, 1923.
17. Reschad, H., and Schilling-Torgau, V., *Münch. med. Woch.*, 1913, lx, 1981.
18. Schlenner, F., *Deutsch. med. Woch.*, 1921, xlvii, 6.
19. Katsunuma, S., *Intrazelluläre Oxydation und Indophenolblau-synthese*, Jena, 1924.
20. McJunkin, F. A., *Arch. Int. Med.*, 1925, xxxvi, 799.
21. Sato, A., and Yoshimatsu, S., *Am. J. Dis. Child.*, 1925, xxix, 301.
22. Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A., *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 14.