BLOOD–PLATELET AND MEGALOKARYOCYTE REACTIONS IN THE RABBIT.[1]

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To one who has had the opportunity of examining the preparations of Dr. J. H. Wright, the last word seems to have been said on the subject of the origin of the blood-platelets and the function of the bone-marrow giant cell, the megalokaryocyte. Yet his published conclusions, that the blood-platelets are portions of giant cell protoplasm constricted off from projected pseudopodia, seem as yet not to have received general acceptance and confirmation, and the earlier views as to the nature and origin of platelets still dominate medical literature touching upon the subject. In consequence, the following study of the megalokaryocytes and of the platelets is offered. An investigation of the relation of the megalokaryocyte to changes in other bone-marrow elements was in progress at the time of Wright's[2] publication, but its completion has been unavoidably delayed, and in resuming it, the author has directed his attention especially to platelet relations, as a result of Wright's discovery.

In taking up the problem of the origin of the platelet the first question to be settled is, what is a platelet? If one follows the view of Schwalbe,[3] he is obliged at the outset to admit that platelets can have no single mode of origin, for he says: " For the study of platelets there is no more appropriate material than the human thrombus. There one finds all forms of blood-platelets, those with and those without the inner body, platelets that stain *in toto* with hematoxylin and those that stain entirely with eosin, platelets that contain more

[2] *Boston Med. and Surg. Jour.*, 1906, cliv, 643.

[3] *Ergebnisse der allg. Pathol.*, 1907, xi, ii, 919.

or less clearly hemoglobin and those without a trace of hemoglobin." Such a collection of cell fragments is surely heterogeneous; yet, except that it probably includes platelets, such a mass bears no relation to the platelet found in the normal circulating blood. It requires but one carefully and quickly made blood smear from human or animal source, stained by Wright's stain, to show that the blood contains no such varied group of bodies. On the other hand, except for red blood cells and leukocytes it contains but a single element, which always stains the same—the body for which the name platelet has been appropriated. The platelet shows constantly a definite and characteristic picture, a sharply outlined light-blue body mass in which lie a variable number of granules staining metachromatically, a purple in which the red predominates. No platelet stains with eosin, none takes a deep methylene blue nuclear stain, none shows a trace of hemoglobin. The platelets vary much is size, but not in staining reactions. If one checks his stained smear by examination of a fresh blood specimen in sodium metaphosphate, sodium citrate or sodium fluoride mixture, he finds just as many delicate, refractile, oval or lenticular bodies as he has stained platelets (within the limits of experimental error). This is the platelet dealt with in the following experiments and not the cell fragments to be found in every disintegrating thrombus mass, described by Schwalbe.

The experiments here reported have had as their object the determination of the relation between changes in the platelets and changes in the other blood elements in the circulation, and further, to ascertain whether or not the platelet changes followed any law comparable to those governing leukocytic or red cell variations. The rabbit has been the animal used in this work, chiefly because of the ease of doing blood counts on it, and also because of the author's familiarity with blood reactions in that animal. In the experiments careful erythrocyte and leukocyte counts have been made and then the platelet count obtained by the indirect method, *i. e.,* by ascertaining their number relative to that of the red blood cells in fresh preparations and checking this result by the relative number in carefully made stained smears. As a counting medium for platelets I have used ten per cent. metaphosphate of soda solu-

tion, 0.5 per cent. sodium citrate, and 0.2 per cent. sodium fluoride, all of which give parallel results in my experience. The first apparently preserves the platelets the best, if a preparation is obtained free from phosphoric acid. Although the solutions have been checked against each other, to avoid a possible source of error, a single solution has been used throughout a series of counts. In obtaining the preparation for counting, the rabbit's ear has been shaved, cleaned with alcohol, a drop of the counting solution transferred to the ear by a platinum loop, and a small vein pricked through the drop. The mixture of blood and counting solution is transferred by the loop to the slide, where it is mixed with more of the solution previously placed in the slide, is covered, ringed with vaseline and immediately counted under high power. The author has found a high power dry lens combination (Zeiss obj. 4 mm. oc. 12) preferable to the oil immersion series, as the adhesion of the oil to the lens and cover glass it apt to produce motion among the cells of the preparation. In the counts here recorded, the number of platelets has been determined from the number observed while counting approximately five hundred red cells.

The experimental error by this method in the author's hands has been found to be less than 50,000 platelets per cu. mm. This may seem a high error, but it is one which it almost negligible, being but about 6 per cent. of the total count in the normal rabbit and an error which would cause but little variation in the curves of platelet changes produced in the experiments. Throughout the experiments painstaking efforts have been made to obtain accuracy in all counts. By this technique, the platelets of the normal rabbit have been found to number between 600,000 and 800,000 per cu. mm. and the majority of the author's series have given a count nearer the upper figure; that is, above 700,000.

If there exists any parental relation between red blood cell and platelet, it seems to the author that this relation should be shown in the circulating blood, either during regeneration of red cells or following their destruction. To determine this, rabbits were subjected to an initial bleeding and blood counts made at intervals following. The reaction is shown by the following experiment:

RABBIT A-iii (BELGIAN).

April 27, 1908	R.b.c. 5,416,000	w.b.c. 14,750	platelets 730,000
	Bled 30 c.c. from ear vein.		
April 28, 1908	R.b.c. 4,736,000	w.b.c. 12,250	platelets 580,000
April 29, 1908	R.b.c. 4,904,000	w.b.c. 17,250	platelets 940,000
May 1, 1908	R.b.c. 4,760,000	w.b.c. 19,000	platelets 1,080,000
May 5, 1908	R.b.c. 5,368,000	w.b.c. 9,125	platelets 800,000
May 7, 1908	R.b.c. 5,752,000	w.b.c. 13,000	platelets 750,000
	Animal killed.		

A similar reaction is shown by Rabbit A–iv.

RABBIT A-IV (MIXED WHITE AND BROWN).

Wt. 2,000 gm.

Jan. 7, 1909	R.b.c. 5,280,000	w.b.c. 8,000	platelets 600,000
Jan. 11, 1909	R.b.c. 5,280,000	w.b.c. 8,000	platelets 600,000
	Animal bled 20 c.c. from ear vein.		
Jan. 12, 1909	R.b.c. 3,880,000	w.b.c. 12,000	platelets 220,000
Jan. 14, 1909	R.b.c. 3,456,000	w.b.c. 8,000	platelets 1,000,000
	Animal killed.		

In these two experiments we have a sharp and definite reaction on the part of the platelets which, upon studying the figures, appears to bear no parallel relation to either the red cells or the white cells. In both experiments there is a fall in the platelets following the hemorrhage; then an increase to a maximum well above the normal, and in the animal allowed to live (A-iii) a return to approximately the normal number. This maximum, a thrombocytosis, if it may be so called, has occurred in both cases before there is any marked sign of regeneration of the red cells; and in the second case, while the red cell count is in fact still diminishing. Nor is any relation to the leukocytes to be made out in either case. The more typical leukocytic reaction is shown by Rabbit A-iv, a slight leukocytosis the day following the hemorrhage with a quick return to normal, the leukocytosis occurring while the platelets are at their lowest point. In Rabbit A-iii there was a preëxisting leukocytosis, the result of an epidemic nasal infection going through the rabbit pen, which somewhat disturbs the count.

As it had been noticed in an animal which had received repeated doses of saponin intravenously that there was a high platelet count (1,400,000 per cubic millimeter), it was decided to use this drug to

produce blood destruction—a toxic anemia. The results were some-
what unexpected, for the saponin destroys not only the erythrocytes,.
but also the platelets, as shown by the following experiments:

RABBIT S. R. K. (ALBINO).

April 3	R.b.c. 6,696,000	w.b.c. 16,750	platelets	800,000
April 3	Injection of 5 mg. of saponin in ear vein.			
April 4	R.b.c. 3,336,000	w.b.c. 45,000	platelets	120,000
	nucleated r.b.c. 11,000			
April 5	Rabbit died.			

RABBIT S. R. L. (ALBINO).

April 6	R.b.c. 4,968,000	w.b.c. 16,875	platelets	810,000
April 8	Injection of 4 mg. of saponin in ear vein.			
April 9	R.b.c. 2,880,000	w.b.c. 13,780	platelets	140,000
	nucleated r.b.c. 7,300			
April 11	R.b.c. 3,240,000	w.b.c. 17,125	platelets	110,000
April 15	R.b.c. 3,272,000	w.b.c. 11,375	platelets	230,000
April 19	R.b.c. 3,656,000	w.b.c. 7,000	platelets	680,000
April 23	R.b.c. 4,280,000	w.b.c. 8,000	platelets	1,000,000
April 24	Animal killed.			

It is quite apparent from these experiments that destruction of
erythrocytes by saponin does not lead to a direct increase in the
platelets, or if it does, that they are subsequently destroyed by the
drug, and that the high count of platelets originally noted was the·
result of regeneration of platelets, as·evidenced by the gradual in-
crease in Rabbit S. R. L. A point of extreme interest in the regen-
eration in this rabbit is the greater length of time required for the·
platelets to reach their maximum number as compared with the
time required for them to reach the same point after hemorrhage·
(Rabbits A-iii and A-iv)—fifteen days in the first, and three and
four days in the last two. So great a difference cannot depend
merely upon the greater initial reduction in number of platelets. It
must depend, it seems to the author, upon a fact pointed out by him·
in a previous report,[4] and that is that saponin injected intravenously·
produces an injury to the bone marrow, causing necrosis of cells
and tissue and eventually marked scar-tissue formation in the mar-
row. Such an injury could then influence the regeneration of the·
platelets only if they were formed in the marrow.

[4] *Jour. of Exper. Med.*, 1906, viii, 625.

Although no relation could be made out in the experiments between leukocytes and platelets, it seemed necessary to put this to further test and several experiments were performed in which sterile inflammations were produced, by the use of croton oil externally, aleuronat in the peritoneal cavity, and turpentine subcutaneously. The experiments showed uniformly an initial fall in the number of platelets and a secondary rise, where the animal was allowed to live. The following rabbit, in which a relatively large dose of turpentine was injected, producing a most extensive subcutaneous exudate, gave the highest reaction in platelets of the author's series, a reaction verified by repetition of the counts in preparations from different veins in both ears and by the stained smears.

RABBIT A-V (MIXED WHITE AND BROWN).

January 19	R.b.c. 6,280,000	w.b.c.	8,500	platelets	720,000	
January 19	7 minims of turpentine injected subcutaneously					
January 20		w.b.c.	2,000	platelets	430,000[5]	
January 21	R.b.c. 6,944,000	w.b.c.	2,125	platelets	430,000	
January 23	R.b.c. 6,352,000	w.b.c.	6,000	platelets	720,000	
January 25	R.b.c. 6,000,000	w.b.c.	10,700	platelets	1,700,000	
January 26	R.b.c. 6,096,000	w.b.c.	16,000	platelets	1,300,000	

Animal killed.

It will be noted here that there is a reduction in platelets during the early stage of the inflammation, while there is a corresponding leukopenia and an increase in red cells, due to the withdrawal of serum into the inflammatory exudate. This is followed by a thrombocytosis which reaches its maximum and has begun to recede, while the leukocyte count is still on the increase. There is then no parallelism between the leukocytes and the platelets.

It would seem from the uniformity of the counts of the blood elements in these experiments, which have been made with the greatest care for accuracy, that the platelets follow a curve independent of the other blood elements; and that in their regeneration they follow the general pathological law of a regeneration in excess, giving a thrombocytosis comparable to a leukocytosis, or to the erythrocytosis which may be obtained in an animal with dry tissues,

[5] Ratio of platelets and red cells established. Number estimated from red count of succeeding day.

as the rabbit. This independence and uniformity of reaction leads naturally to the conclusion of an independent source of origin and the unity of the platelet group.

The experiments, in so far as the counts are concerned, give no clue as to the origin of the platelets, unless it be given by the saponin experiments in which, after the marrow injury, the regeneration of the platelets is delayed. The evidence of the source of the bodies must be obtained from morphological study of the tissues of the animals used, and from the study of blood smears, appropriately stained. This evidence has already been furnished by Wright, and the author's results are in general agreement with his findings.

The tissues of the animals used in the experiments already cited have been carefully studied for evidence as to the origin of the platelets. Fixation for the routine hematoxylin and eosin specimens has been made in Zenker's fluid; for the application of Wright's special stain, in warm saturated corrosive sublimate solution. Attention has been given especially to the bone-marrow and the megalokaryocytes. From counts made for me some time previously, by Dr. R. V. Lamar, it was evident that the megalokaryocytes are increased in number in certain conditions, particularly in inflammations and in regeneration after hemorrhage or toxic blood-injury. To determine this point, in reference to the platelet supply, counts have been made on the marrows of the animals used in these experiments, and of others in which the blood condition was known. It is impossible that these counts should have mathematical accuracy, for two reasons: first, because of the irregular distribution of the megalokaryocytes in the marrow, and second, because of the difficulty of establishing a normal count. Yet they have greater value than the mere impression gained from a casual observation of the marrow, that the cells are increased or diminished in number. For all counts, marrow has been taken from the middle third of the femur, and they are reckoned for an area of one square millimeter of a section 5μ thick. The normal number of megalokaryocytes appears to be approximately 20 per square millimeter, varying a few either above or below this number in different fields. Rabbit A-v, 7 days after a turpentine injection, and with a platelet count of 1,300,000, showed 53 megalokaryocytes per unit area; Rabbit A-iv,

three days after hemorrhage, with a count of 1,000,000 platelets, showed 47; Rabbit S. R. K., one day after a saponin injection, with platelets 120,000, showed 19; Rabbit S. R. L., 15 days after a saponin injection, with platelets 1,000,000, had 31 per unit area. Counts in animals not included in the earlier part of this report are also of interest. In a rabbit twenty-four hours after an infection with Staphylococcus aureus magalokaryocytes were 12 per unit area: in a second rabbit twenty-four hours after such an injection they were 33; in a rabbit twenty-four hours after an aleuronat peritonitis they were 25; forty-eight hours after a similar injection in another rabbit they were 47, and in another four days after pleural injection they were 37.

When these results are considered, it is apparent that immediately after the establishment of an inflammatory condition or of a toxic anemia in the animal, there is a reduction in the number of megalokaryocytes in the marrow. This reduction is explained by sections from the lung in these cases. It is a fact of common observation that in every case of sharp leukocytic reaction in man a variable and oftentimes large number of megalokaryocyte nuclei are found in the lung capillaries. This is true also of the rabbit. In every case of leukocytosis, and in the reaction to hemorrhage and ricin, and saponin intoxications, a large number of these nuclei are found wedged into the lung capillaries. At times they may get through the lung and may be found in other organs. The demonstration by Askanazy[6] that in the warm stage the megalokaryocyte possesses active amoeboid motion would indicate that this is an active emigration and not a passive washing out or breaking loose of the cells.

The bone marrow deficiency in giant cells thus produced is followed by an increase in their number. Peculiar complex mitotic figures have been found in megalokaryocytes in all of the regenerating marrows studied. Yet I am not ready to answer the question definitely whether these mitotic figures lead to cell division and to an increase in the number of cells, or merely to an increase in the complexity of the nucleus of the cell. At the present stage of my study of the cell, I am inclined to the latter view largely from my failure to find any evidence of either division of the chromatin

[6] Quoted by Schridde, *Anat. Hefte,* 1907, xxiii, 1.

masses or of the cell, while, on the other hand, there are found in these marrows many giant cells with relatively simple nuclei, even merely bi-lobed nuclei. This suggests that these cells develop from a mononuclear cell, and that the complex nucleus of the adult cell is arrived at by repeated but incomplete mitoses. Nevertheless, the occurrence of mitoses in these cells, following the depletion of the marrow, would indicate a heightened activity of these elements.

It may be objected that mere increase in number of megalokaryocytes is not an indication of increased functional activity on their part, and, further, that their increase in number, occurring simultaneously with an increase in the platelets of the circulation, does not necessarily indicate any relation between the two events. This is admitted. Yet in these marrows we find evidence that the giant cells are active in parting with portions of their protoplasm. In fact, we find that a high percentage of them have either no discernible protoplasm or only a narrow rim with ragged outline. For example, in the rabbit A-iv, in the regeneration following hemorrhage, 46 per cent. of the megalokaryocyte nuclei were either without protoplasm, at least as far as could be made out, or had but a slight ragged rim about them. Likewise in Rabbit A-v, in the regeneration after the turpentine inflammation, there were 38 per cent. of such nuclei. This sign of activity is found also in marrows of normal animals, for it is apparently an indication of the normal function of the megalokaryocyte. For the determination of the manner in which the giant cell loses its protoplasm, and the relation to the platelet, specimens of marrow prepared according to the Wright corrosive-acetone-turpentine method and stained by Wright's stain are very satisfactory, although confirmatory evidence is given by Zenker-hematoxylin-eosin specimens. I have not succeeded in obtaining the characteristic staining of the megalokaryocytes in the marrow of the rabbit by the Wright method after methyl alcohol fixation. With corrosive sublimate fixation, however, the staining is usually successful and the picture is definite. The protoplasm of the cell takes a delicate clear blue tint, and in it are imbedded granules with a metachromatic stain. The granules vary in number and distribution. In some cells they are massed about the nucleus, in others they are more or less evenly distributed,

while in still others they tend to be massed toward the periphery and are more or less clumped. The staining reaction is identical with the staining of the protoplasm and granules of the platelets in the circulation when stained in a blood smear with the Wright stain. This identity of staining reaction in the two elements is particularly well brought out in marrow and lung from the same animal carried through the same fixing and staining solutions. In animals with a high peripheral platelet count, the lung capillaries are crowded with platelets, and the observation of them in this situation is rendered easy by the very thin sections one can obtain of the organ. This establishment of the identity in staining reactions of the protoplasm of the megalokaryocytes and its projected pseudopodia and of the platelets is the most valuable feature of the Wright method.

The author has studied with care a series of marrows prepared both by Wright's method and by the Zenker fixation and has found every evidence of the formation and separation of pseudopodia from the megalokaryocyte. These features are most prominent in active marrows, by which is meant marrows taken from animals while the platelet count was increasing or at its maximum, and they are most evident in marrows fixed as soon as possible after the death of the animal. I can understand Schridde's failure to confirm Wright's findings only from the fact that he used chiefly human material obtained at autopsies performed some time after death. In the rabbit these megalokaryocyte projections or pseudopodia vary much in size. One finds short projections of about the diameter of an ordinary platelet, but more often they are from two to three times that width and of considerable length. One can find also every stage of the constricting off process from its inception to its completion with entire separation of the mass. In the highly active marrows, the pseudopodia are often of considerable size, even approaching half of the cell volume. One remarkable picture was seen in which such a large mass was still connected with the protoplasm surrounding the nucleus by a long drawn-out slender neck, while at its distal end it was breaking up into masses of platelet size. Slender pseudopodia of great length were found in the marrow capillaries, and in one animal similar large masses were found in the lung capillaries, one with an area approximately three times

that of a red cell and showing a central constriction as if in process of segmentation. This mass was clearly not the result of fusion of platelets, as was shown by the arrangement of the granules in it, by the sharp outline and by the fact that the platelets in this lung specimen existed as clear cut individuals and not as fused masses. It is apparently by the stripping off of such large protoplasmic masses that there is left in the active marrows so high a percentage of almost naked megalokaryocyte nuclei.

These marrow and lung findings correspond well with what has been noted constantly in the fresh and stained preparations of the circulating blood made from the animals used in these experiments. The observation has been that in the animals with high counts after a rapid rise in platelets, the platelets have shown a great variation in size with a tendency to a large average diameter and with the occurrence of some very large forms. A recently examined blood smear from a case of Hodgkin's disease, in which there was an unusual number (1,140,000) of platelets,[7] showed that this variation in size of platelets and the occurrence of large forms is not confined to animals, but may take place also in human beings. This smear was very successfully made from the platelet standpoint and showed them well preserved, with sharp outlines and not fused into masses. The size of the platelets was very variable. Some were long and narrow, others more oval. Two of the long, narrow platelets were fully 15 μ in length, and one oval form noted especially was of similar length and of a diameter almost equal to that of a red cell. From the sharp cut outlines of these platelets and from the distribution of the granules, there was not the slightest possibility that they were masses of fused platelets.

As a result of this study of the platelets and the megalokaryocytes the author feels justified in drawing the following conclusions:

1. The platelets of the circulation form a group of elements of uniform structure and of common origin.

2. In their regeneration after destruction or loss from the circulation they follow the usual pathological law of regeneration in excess, producing a thrombocytosis comparable to a leucocytosis.

[7] The discussion of platelet relations in Hodgkin's disease is reserved for a subsequent report.

3. This curve of regeneration shows no parallelism to that of the erythrocytes or of the leucocytes.

4. Synchronous with or preceding the appearance of an increased number of platelets in the blood stream, the megalokaryocytes of the bone marrow are increased in number.

5. The megalokaryocytes, by separation of pseudopodia of various sizes, become reduced to almost naked nuclear masses.

6. These pseudopodia are identical in staining reactions with the platelets, and do in fact form them by further segmentation.

7. The megalokaryocyte is the only source of the blood platelets.