THE ORIGIN OF PLATELETS

Their Behavior in the Heart-Lung Preparation

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Considerable evidence is available to justify the conclusion that platelets originate from megakaryocytes (Tocantins, 1). But it has been questioned whether the megakaryocytes of the bone marrow or of the lungs are responsible for the greater production of platelets. Howell and Donahue (2) raised the question and have concluded that the lungs are the principal site of formation of platelets. They based their conclusions chiefly on the following observations, (*a*) that the platelet count is higher in arterial than in venous blood and that this difference is even greater if platelets are first reduced by some experimental procedures, and (*b*) that when isolated lungs are perfused with a solution which tends to preserve the platelets, the effluent shows an increasing proportion of platelets to red cells as the perfusion proceeds.

It occurred to us that the Starling heart-lung preparation might with advantage be employed to determine the platelet productivity of the lungs. We were encouraged in this idea by our observation in the first experiment that there is a very rapid reduction in the platelets soon after the preparation is made. This recalled Howell's contention that when the platelet count is decreased by various means, platelet production by the megakaryocytes of the lungs is most rapid. Further, if platelet production could be demonstrated in such a simple preparation it would be a relatively easy matter to study in more detail the conditions which govern it.

Methods

Heart-Lung Preparations.—Dogs weighing between 9 and 14 kilos were used. After fasting for about 24 hours they were anesthetized with ether and the heart-lung preparation made in the usual way. In the external circuit the blood flowed through a few short lengths of glass tubing, about 6 feet of rubber tubing, some surgical drainage tubing to serve as peripheral resistance, and into a small cylinder fitted with a stopcock, used occasionally to measure the rate of flow, and below this into a 250 cc. cylinder which served as a reservoir. It was the practice to give this external circuit a final wash with Ringer's solution immediately before use. It will be readily seen that the

blood flows over an extensive foreign surface which might be expected to produce considerable platelet damage. Usually about 200 to 300 cc. of blood were used and the rate of flow varied between 80 and 120 cc. per minute. Air was used in the artificial ventilation except in one experiment when air containing 10 per cent CO_2 was employed without any notable difference. Unless otherwise stated, the animal's own blood was used and allowed to flow directly into the external circuit.

Platelet Counting Technique.—Blood samples were taken through the rubber tubing near the heart, except as otherwise stated. The sampling fluid was the formalin-citrate mixture recommended by Tocantins (3), but without any dye. The citrate and formalin were stored in separate bottles in the refrigerator, and the dilution of the formalin and its addition to the citrate was made on the day it was required. This mixture was filtered and when kept in the refrigerator could be used a day or two later, but as a rule a fresh mixture was made up for each experiment. The syringes were of ½ cc. capacity, and each was calibrated with its own 27 gauge needle. They were washed in water, alcohol, and ether, and after drying were chilled in the refrigerator along with the sampling fluid. When a sample was to be taken, this fluid was drawn through the needle of the syringe, adjusted to the 0.4 cc. mark with the expulsion of any air bubbles, and then 0.2 cc. was delivered into a small test tube. The needle was then inserted into the blood stream and a sample of 1/10 cc. of blood withdrawn. This was immediately expelled into the test tube beneath the surface of the sampling fluid and mixed by passing it back and forth in the syringe. The test tube was then given a gentle mechanical shaking for several minutes. It was found that these samples could be left for 24 hours in the refrigerator without any significant alteration of the platelet count, although generally the counts were made within a few hours after the removal of the blood.

Because of the sampling fluid present in the needle, the dilution of the blood in the syringe was about 1 in 5.5. For counting the red cells, the samples were further diluted in the usual 1/100 pipette, giving a final dilution of 1 in 550. This count was made immediately after a drop was mounted in the counting chamber and could be completed without contact hemolysis becoming a disturbing factor. When the platelet count was low, its determination at this dilution was unreliable. It was found that a 10 per cent solution of urea, such as employed by Lempert (4), when used as a diluting fluid in the 1/10 pipette gave a mixture in which practically all red cells had disappeared, while the platelets, previously fixed by the formalin of the sampling fluid, were left slightly swollen but easily recognizable. The time required for the platelets to settle in this urea dilution was slightly longer than in the sampling fluid. Four chambers were counted with each blood sample.

RESULTS

The results of 12 heart-lung experiments are shown in Table I. Since it is impossible to take 1/10 cc. of blood in the syringe with absolute accuracy, it is customary to note the relation of the platelets to the erythrocytes in order to make certain that any variation in platelet count is not due to a variation in the size of the blood sample. In place of an erythrocyte/platelet ratio, we prefer adjusting the platelet counts in any one experiment to the same basis of red cells, usually the number (underlined in the table) contained in the first or second sample. Since no hemolysis has been visible

in the samples when the red cells were allowed to settle out, and since any variation of concentration of the blood should not alter the relation of the cellular elements one to another, this adjustment seems a more satisfactory way to present the true numerical changes in the platelets. The samples are numbered in the table in the order in which they were taken. Arterial and venous samples could not be taken simultaneously, but where they are compared they were taken in immediate succession about 1 minute apart. The comparison is shown in the two columns in the middle of the table.

Clumping and Reduction of Platelets.—It will be seen that the platelet count of the blood circulating in the preparation shows a rapid and marked decrease from that obtained immediately before operation or even just before the blood was allowed to pass into the external circuit. The immediate effect of the foreign surface on the platelets was determined in the following way: The usual procedure was followed of filling with Ringer's solution the tubing connecting the reservoir with the venous cannula. Blood was allowed to flow into the reservoir until the desired volume was obtained. The clamp on the venous tubing was then removed, and simultaneously the aorta was securely tied, thus completing the preparation.

In Experiment VI, at the end of 1 minute and 2 minutes from the commencement of the flow, samples of blood were taken from the rubber tubing just as the blood passed into the measuring cylinder. Up to this time the venous tubing was clamped. The counts show the rapidity with which the platelets decreased although only about one-half of the external circuit had been traversed. In Experiment VII the sample at 1 minute was taken under the same conditions, while that at 2 minutes was taken from the rubber tubing leading back to the heart about 10 seconds after the clamp was removed from this tubing, *i.e.*, after the external circuit had been established. This last sample therefore shows the effect on the platelets of the passage of the blood around nearly the whole external circuit with very temporary stagnation in the reservoir. It will be seen that there is a reduction to about 1/3 of the original count in this 2 minute period. All other samples were taken near the heart, as previously stated, and the lowest values were obtained within a few minutes of establishing the external circuit. It is noteworthy that in spite of the considerable foreign surface to which the blood is subjected the platelets do not completely vanish, and most of those that remain unclumped are normal in appearance. There is no ready explanation for this difference in behavior of the unclumped platelet.

Sometimes heparin was added to the blood as it flowed into the external circuit, and except for a few units added to the blood near the cannulae,

TABLE I

Sample No.	Time taken	Vein				Artery				
		R.B.C. × 10^6	Platelets × 10^3	Clumped	Adjusted count	Adjusted count	Clumped	Platelets × 10^3	R.B.C. × 10^6	Sample No.
				per cent			*per cent*			
Experiment I										
1	Before operation	7,175	491	—	—	—	—	—	—	—
2	17 min. after circulation established	6,274	55	—	55	50	—	49	6,163	3
4	37 " " " "	6,656	93	—	88	70	—	71	6,554	5
Experiment II										
1	Before operation	6,620	277	—	—	—	—	—	—	—
2	12 min. after circulation established	5,668	72	—	72	97	—	101	5,891	3
4	48 " " " "	5,762	78	—	77	95	—	95	5,654	5
6	1 hr. 23 " " " "	5,834	127	—	123	118	—	118	5,638	7
8	2 hrs. 18 " " " "	6,599	211	—	187	247	—	275	6,266	9
10	2 " 54 " " " "	6,055	283	—	265	255	—	269	5,975	11
Experiment III										
1	Before operation	5,657	531	—	—	—	—	—	—	—
3	15 min. after circulation established	4,481	34	—	34	46	—	46	4,483	2
5	1 hr. " " "	5,045	55	—	49	39	—	40	4,593	4
7	2 hrs. " " "	4,595	75	—	74	43	—	50	5,178	6
Experiment IV										
1	Before operation	7,850	236	—	—	—	—	—	—	—
2	15 min. after circulation established	7,175	39	—	39	37	—	36	6,968	3
4	1 hr. " " "	7,047	43	—	44	118	—	112	6,825	5
6	1 " 25 " " " "	7,078	79	—	80	64	—	66	7,451	7
Experiment V										
1	Before operation	7,117	484	—	484	—	—	—	—	—
2	10 min. after heparin	6,550	424	—	461	—	—	—	—	—
3	15 " after circulation established	6,740	19	—	20	43	—	41	6,800	4
5	1 hr. " " "	6,686	114	—	121	156	—	145	6,620	6
Experiment VI										
1	Before operation	8,076	321	—	321	—	—	—	—	—
	1 min. after circulation established	—	—	—	—	239	—	245	8,250	2
	2 " " " "	—	—	—	—	186	—	201	8,717	3
	19 " " " "	—	—	—	—	26	—	24	7,417	4
5	1 hr. 3 " " " "	7,638	71	—	75	82	—	73	7,182	6
Experiment VII										
1	Before operation	7,304	318	—	318	—	—	—	—	—
	1 min. after circulation established	—	—	—	—	175	25	165	6,886	2
3	2 " " " "	6,104	79	85	95	—	—	—	—	—
4	6 " " " "	5,935	8	64	10	8	38	6½	6,095	5
6	26 " " " "	5,650	20	52	26	24	58	20	6,024	7
8	1 hr. 5 " " " "	6,360	48	49	55	37	36	33	6,440	9
10	1 " 51 " " " "	6,401	90	27	103	103	27	82	5,820	11
12	2 hrs. 31 " " " "	5,917	101	23	125	114	13	97	6,233	13

TABLE I—*Concluded*

Sample No.	Time taken	Vein				Artery				
		R.B.C. × 10^3	Platelets × 10^3	Clumped	Adjusted count	Adjusted count	Clumped	Platelets × 10^3	R.B.C. × 10^3	Sample No.
				per cent			*per cent*			
Experiment VIII. Perfusion with platelet-free blood										
1	Before operation	6,171	168	—	—	—	—	—	—	—
2	7 min. after circulation established	5,659	16	0	16	15	0	14	5,129	3
4	45 " " " "	5,308	13	0	14	18	0	17	5,378	5
6	1 hr. 30 " " " "	5,070	13	0	14	15	0	14	5,136	7
8	2 hrs. 11 " " " "	5,287	15	0	16	17	0	17	5,668	9
Experiment IX. Platelets reduced by centrifuging the blood used for perfusion. 10 per cent CO_2										
1	Before operation	6,480	379	—	379	—	—	—	—	—
2	5 min. after circulation established	6,143	17	39	18	22	62	22	6,608	3
4	50 " " " "	6,913	36	53	34	31	37	31	6,565	5
6	1 hr. 30 " " " "	6,675	55	23	53	52	26	53	6,551	7
8	2 hrs. 30 " " " "	7,016	55	15	51	46	6	50	7,022	9
10	3 " 30 " " " "	6,581	50	15	50	49	5	49	6,482	11
12	4 " " " " "	7,106	47	8	43	43	6	45	6,773	13
Experiment X. Platelets reduced by return of defibrinated blood before operation										
1	Before bleeding	6,482	320	—	320	318	—	338	6,879	2
3	After "	6,876	142	—	134	117	—	126	6,984	4
5	15 min. after circulation established	6,475	22	23	22	19	10	20	6,809	6
7	1 hr. " " "	6,136	28	9	29	32	9	33	6,669	8
9	2 hrs. " " "	6,061	39	11	42	62	9	61	6,381	10
11	3 " " " "	6,034	56	5	60	57	2	54	6,136	12
13	4 " " " "	6,468	55	2	55	65	7	65	6,452	14
Experiment XI. Circulation 10 min. through right lung only, then tied; circulation opened through left lung										
1	Before operation	6,577	398	—	398	—	—	—	—	—
2	10 min. after circulation established	5,783	8	55	9	15	35	14	5,912	3
4	1 hr. " " "	6,304	38	23	40	45	17	46	6,676	5
6	2 hrs. " " "	6,462	48	4	49	48	2	49	6,774	7
8	2 " 40 " " " "	6,691	44	3	43	44	0	40	6,046	9
Experiment XII. Heparin for 1 hr. before establishing external circuit										
1	Before operation	4,941	274	—	274	—	—	—	—	—
3	19 min. after circulation established	4,389	86	48	97	81	65	79	4,846	2

none was injected into the animal. At other times (Experiments II, III, and VI) the heparin was injected into the animal about 3 minutes before allowing the blood to pass to the external circuit. In Experiment V the heparin was allowed to circulate within the animal for 10 minutes. In all these experiments, in which the amount of heparin used was such as to

give a concentration of about 3 units per cc. of blood in the preparation, the platelets were much reduced and clumped. A larger dose of heparin (5 units per cc.) and for a longer time (1 hour) was tried in Experiment XII, but again the platelets were clumped. It seems evident that a distinction must be drawn between clumping of the platelets in the blood stream as we have observed, and the clumping on a damaged or foreign surface, *i.e.*, the formation of a thrombus, for while heparin in sufficient amount and given sufficient time prevents the latter (Solandt and Best, 5), the former still occurs.

Recovery of the Platelet Count.—In Experiment II the number of platelets decreased initially, though not to the usual extent, and then progressively increased until the count was as high as that obtained in the intact animal immediately prior to the operation. This result, at the time, appeared to us to give strong support to the conclusions of Howell and Donahue. Experiments III to VI, though not showing as good a recovery of the platelets, do show an increase over the first low values.

It was then decided to determine not only the number of platelets, but also the percentage of them which occurred in clumps. Where platelets adhered in groups of 3 or over they were regarded as clumped. Usually the clumps did not exceed 6 or 8 platelets, but occasionally clumps were much larger (30 to 50) and at such times accurate counting was, of course, impossible. However, it will be seen that in Experiments VII, IX, X, and XI as the platelet count increased the extent of clumping showed a corresponding decrease. When very few clumps could be observed the platelets appeared to have reached their maximum number. These facts are of considerable interest. While they may be interpreted as indicating a removal of clumps from the circuit with new production of platelets, presumably by the lungs, an alternative explanation is forthcoming. It is that the increase in the number of circulating platelets is due to the gradual sweeping out of platelets adhering to the walls of the pulmonary vessels by the perfusing heparinized blood and possibly to the disintegration of some clumps thus releasing apparently undamaged platelets. Certainly one is impressed with the normal appearance of the platelets of the later samples. Also the bigger clumps are usually seen in the early samples while only small clumps occur in the late samples.

Perfusion with Blood in Which Platelets Had Been Previously Reduced.—In Experiment VIII platelet-free blood was prepared from heparinized dog's blood which had been stored for a few days in the refrigerator. The red cells were well washed in the centrifuge with Ringer's solution immediately before use, and the plasma was given a prolonged centrifugation. Some of this platelet-free blood was then allowed to flush out the heart and

lungs and flow to waste. The remainder (about 250 cc.) was used to maintain the preparation. Of the red cells in circulation in this preparation 90 per cent were crenated. It was presumed that the 10 per cent normal looking red cells were host cells not washed out by the preliminary perfusion. Further it is seen that the platelet count is reduced to about 10 per cent of that obtained in the animal prior to operation. It thus appears that 10 per cent of the animal's own blood remained in the circulating blood of the preparation. No clumping of the platelets was observed and for over 2 hours no significant change in their number occurred. Experiment IX was performed in a slightly different manner. Stored blood was rendered platelet-free as in Experiment VIII and used to flush out the heart and lungs. Then heparinized blood, freshly obtained from another dog, was rendered platelet-free and used in the preparation. It will be seen that in this experiment there was appreciable clumping of the platelets, and that with the decrease in the extent of clumping there was an increase in the platelet count. But in spite of the length of the experiment the rise in platelets was relatively small and the samples at 4 hours showed a lower count than at $1\frac{1}{2}$ hours.

In Experiment XI a few minutes after the chest cavity was opened, a bulldog clamp was placed on the branch of the pulmonary artery to the left lung. After the external circuit had been established for 15 minutes this clamp was removed and the right lung completely clamped. In this way it was presumed that the numerous clumps which form early in the first few minutes of the preparation would be caught in the right lung and removed from the blood while the circulation could continue through the relatively clump-free lung only. Certainly in this experiment the remaining clumps in the circulation disappeared relatively quickly and the platelet increase was not very large.

In performing Experiment X it was thought that disintegration of platelets would provide a stimulus for platelet production within the animal. The femoral artery of the anesthetized animal was exposed and cut. The animal was bled several times from the proximal end of the artery and each lot of blood after being defibrinated and filtered was reinjected into the distal end. In this way the platelet count was reduced to about one-half The heart-lung preparation was then made, using the animal's own blood exclusively. Clumping was comparatively slight and the platelet count did not increase during the last 2 hours of the perfusion.

DISCUSSION

During the first few minutes of the circulation of the heparinized blood in the heart-lung preparation there is a considerable decrease in the platelet

count. Later the count increases. In one experiment this subsequent increase reached 95 per cent of the original platelet count of the intact animal. The majority of the platelets seen in these later samples are normal in appearance. These results appear to provide affirmative evidence for platelet production in the lungs.

But other results which we have secured in these preparations make it extremely doubtful whether any new formation of platelets occurred. When the platelet count is low most of the platelets are clumped. As the count increases the clumps disappear. When the clumps become few in number the total count ceases to increase. Also in those preparations containing blood freed beforehand of many of its platelets, the subsequent platelet rise is much less evident. Again, if one supposes a continuous destruction of platelets in the external circuit of the preparations, then platelet production in the lungs would be reflected in a higher platelet count in the blood entering the external circuit than in that leaving it. This is not so. The venous count was higher 21 times, the arterial 18 times, and twice the counts were equal. The average of these counts shows no significant a/v difference. The latter result is in accord with that of Tocantins and Bradshaw, reported by Tocantins (1), who were unable to obtain the higher arterial than venous counts of Howell and Donahue.

The finding of Howell and Donahue of a higher platelet count in blood passing from the lungs than when entering them is very difficult to explain unless one accepts their conclusion of platelet formation in these organs. But such a high rate of platelet production is surely unlikely. The average a/v difference of the pulmonary circuit which these workers obtained was 9 per cent of the average venous platelet count. The amount of blood which flowed through the lungs each minute would not be very different from the total blood volume of the animal. An a/v difference of 9 per cent would thus indicate that the circulating platelets of the animal were completely renewed every 11 minutes or so. Even an a/v difference of about 3 per cent, which they considered to be the smallest significant difference due to inherent limitations of the counting technique, would still indicate an unreasonably high rate of production. Certainly the other experimental evidence that when the isolated lung is perfused, the perfusate becomes relatively richer in platelets than in red cells, may mean that many platelets adhere to the walls of the pulmonary capillaries and that they are washed out more slowly than are the red cells.

The heart-lung preparation, as we have used it, may be unsuitable for a study of the possible production of platelets by the lung tissue. While it is true that these experiments have been conducted for relatively short periods, this disadvantage is partly compensated for by the fact that the

amount of blood circulating through the preparation was usually ¼ or less of that of the intact animal. The products of platelet breakdown may form a stimulus to production, but they may not act directly upon the megakaryocytes but through some intermediary substance. In this connection it should be mentioned that Toyoda (6) claims to have demonstrated a hormone produced in the spleen which causes a thrombocytosis. Torrioli and Puddu (7) also have shown that small doses of a protein-free aqueous extract of spleen can stimulate megakaryocytes in cultures of bone marrow and produce an appearance suggestive of platelet formation. If such a hormonal mechanism be necessary for platelets, then the heart-lung preparation would be unsuitable for demonstrating production unless the hormone be also supplied. Osgoode and Brownlee (8) have made the interesting observation that in cultures of bone marrow a citrated plasma is necessary for the cultivation of cells of the thrombocyte series, although serum will suffice for all other types of cells. Defibrinated blood may thus exert an inhibiting effect upon platelet production.

SUMMARY

1. In heart-lung preparations perfused with heparinized blood, clumping of platelets and a sharp fall in the platelet count occurred in the first 5 to 15 minutes after the external circuit was established.

2. In 10 such preparations surviving from 1 to 4 hours, only one showed a return of the platelet count to the level existing before operation.

3. When platelets were reduced in numbers before the blood was used for perfusion, the count tended to level off after a small recovery, if any.

4. An estimate of clumping showed it to bear an inverse relationship to the platelet recovery.

5. No satisfactory evidence of platelet production in the lungs has been obtained in these studies with heart-lung preparations.

6. The possibility of special factors which may influence platelet formation is discussed.

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