BLOOD - PLATELET ANTI-SERUM, ITS SPECIFICITY AND RÔLE IN THE EXPERIMENTAL PRODUCTION OF PURPURA.*

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Part II.

IN a previous communication (Bedson, 1921¹), it has been shown that an anti-platelet serum contains an antibody specific for platelets, it being possible by means of absorption to remove all red-cell antibody from such a serum and to leave the specific antibody untouched. In the same communication mention was made of the investigations carried out with regard to the production of purpura in animals (Ledingham (1914²), Ledingham and Bedson (1915³)), and it will be recalled that this phenomenon was produced by anti-platelet serum alone, neither anti-red cell serum nor anti-leucocyte serum being able to do so. Now the serum with which we then worked agglutinated both platelets and red cells, the respective titres of the anti-guinea-pig platelet serum being 1 in 128 (*v. platelets*) and 1 in 2048 (*v. red cells*). *In vivo* also this serum produces hæmagglutination, this phenomenon occurring simultaneously with the onset of purpura. In view of these observations it was thought that most probably the hæmagglutinin present in anti-platelet serum played an important rôle in the production of the hæmorrhages, the masses of agglutinated red cells held up in the capillaries leading to their rupture. Obviously this was not the only factor concerned, since an anti-red-cell serum, though capable of giving rise to hæmagglutination *in vivo*, did not produce purpura. Since it has been shown possible to remove the red-cell antibody from an anti-platelet serum, leaving untouched the platelet antibody, it was decided to test such an absorbed serum *in vivo*. The interest of this experiment is obvious. It should give us a clearer insight into the mechanism of the hæmorrhage, which up to now has remained a matter of pure conjecture, and incidentally it should throw further light on the functions of the platelet.

IN VIVO EFFECTS OF SERA PREPARED AGAINST BLOOD ELEMENTS OF GUINEA-PIG.

It was decided first of all to test *in vivo* the various sera, prepared against the blood elements of the guinea-pig, making observations at intervals on the blood picture, particularly with reference to the platelets, and noting also the presence or absence of hæmorrhages.

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This was a repetition of our earlier work, at any rate as far as the anti-platelet, anti-red cell, and anti-leucocyte sera were concerned. Finally, the absorbed anti-platelet serum was to be tested *in vivo*, noting particularly its power to give rise to hæmorrhages.

Technique of the Platelet Count.

The number of red cells and leucocytes was estimated by means of the Thoma-Zeiss pipettes and counting chamber. The following method was used in counting platelets. The ear of the guinea-pig was cleaned up with ether and a large drop of 2 per cent. citrate in saline deposited over a suitable vein. The vein was then opened *through* the drop of diluent by means of a sharp needle, the blood flowing directly into the diluent. With a platinum loop of convenient size a drop of this citrated blood was mixed on a slide with an equal quantity of a solution of brilliant cresyl blue (1 per cent. aq. sol. brilliant cresyl blue 1 part, 2 per cent. citrate in saline 6 parts) covered with a cover slip and the preparation ringed with paraffin. A sufficient quantity should be taken to spread out evenly under the cover slip without causing it to float. A satisfactory preparation must show no clumping of the platelets. The platelets (stained blue) and the red cells were then counted in a number of fields of this preparation and the ratio of platelets to red cells calculated. The number of red cells per cubic millimetre having been estimated in the usual manner, the absolute number of platelets per cubic millimetre is readily arrived at.

Experimental Data.

The findings in the guinea-pigs inoculated with the various anti-sera are tabulated below:—

TABLE I.
Anti-platelet Serum.

Time of Observation.	Red Cells	Platelets.	Platelet/Red Cell Ratio.
Before inoculation	6,496,000	1,160,000	1 to 5·6
1·0 c.c. <i>Anti-platelet Serum</i> inoculated intraperitoneally.			
Six hours after inoculation	6,638,000	57,000	1 to 111

Animal killed with chloroform six and a quarter hours after inoculation.

Post-mortem Findings.—Skin and subcutaneous tissue show a few hæmorrhages: one or two small hæmorrhages in muscles of thigh.

Peritoneal Cavity.—Hæmorrhages in mesentery and in both large and small intestine: small hæmorrhages in epididymis. Liver and spleen appear normal: no macroscopic change in kidney or suprarenal.

Pleural Cavity.—Lungs show a few hæmorrhages: otherwise normal.

TABLE II.
Anti-red-cell Serum.

Time of Observation.	Red Cells.	Platelets.	Platelet/Red Cell Ratio.	Leucocytes.
Before inoculation	5,456,000	900,000	6·0	9,400
1·0 c.c. <i>Anti-red-cell Serum</i> inoculated intraperitoneally.				
Five hours after inoculation	5,560,000	1,010,000	5·5	20,000

Five hours after inoculation hæmagglutination was present. There were no skin hæmorrhages and bleeding time was normal. The animal died during the night.

Post-mortem Findings.—No hæmorrhages in skin or subcutaneous tissue.

Peritoneal Cavity.—Contains some blood-stained fluid : liver enlarged : gall bladder greatly distended with thin reddish bile : spleen enlarged and dark purple in colour : bladder contains blood-stained urine : kidney and suprarenals appear normal : no hæmorrhages anywhere.

Pleural Cavity.—Lungs congested, no hæmorrhages : heart contains laked blood.

TABLE III.
Anti-leucocyte Serum.

Time of Observation.	Red Cells.	Platelets.	Platelet/Red Cell Ratio.	Leucocytes.	Differential.					Remarks.
					Polymorph.	Eosinophile.	Lymphocyte.	Mononuclear.	Mast Cell.	
Before inoculation 1.0 c.c. <i>Anti-leucocyte (polymorphonuclear) Serum</i> inoculated intraperitoneally.	5,520,000	745,000	7.4	4600	51.3	4.0	38.0	5.3	1.3	
Four hours after inoculation	5,920,000	730,000	8.1	1000	14.0	...	73.0	12.0	1.0	No skin hæmorrhages. Degenerate polymorphs in blood smear.
Twenty - four hours after inoculation	4,336,000	522,000	8.3	2400	0.5	1.0	92.5	6.0	...	No skin hæmorrhages. Hæmagglut. + Bleeding time normal.

Animal killed with chloroform after 24-hour observation.

Post-mortem Findings.—No hæmorrhages in skin or subcutaneous tissue.

Peritoneal Cavity.—Congestion of all viscera : no hæmorrhages : hæmagglutination in mesenteric vessels very marked : spleen somewhat enlarged and dark in colour : liver shows no macroscopic change.

Pleural Cavity.—Lungs, no hæmorrhages. Heart, contains agglutinated blood.

TABLE IV.
Anti-serum (precipitating) Serum.

Time of Observation.	Red Cells.	Platelets.	Platelet/Red Cell Ratio.	Leucocytes.	Remarks.
Before inoculation . . .	5,440,000	850,000	6.4	7400	
1.0 c.c. <i>Anti-serum (precipitating) Serum</i> inoculated intraperitoneally.					
Five hours after inoculation	5,200,000	800,000	6.5	7800	Bleeding time,—normal. No hæmagglutination. No purpura.
Twenty-four hours after inoculation	5,440,000	891,000	6.1	9220	No change.

TABLE V.
Anti-whole-blood Serum.

Time of Observation.	Red Cells.	Platelets.	Platelet/Red Cell Ratio.	Leucocytes.	Differential.				
					Polymorph.	Eosinophile.	Lymphocyte.	Mononuclear.	Mast Cell.
Before inoculation	5,920,000	768,000	7·7	8,400	47·0	...	42·0	10·0	1·0
1·0 c.c. <i>Anti-whole-blood Serum inoculated intraperitoneally.</i>									
Fifteen hours after inoculation	640,000	66,000	9·6	40,000	66·0	2·5	20·5	11·0	...

At the 15-hour observation—Hæmagglutination +, bleeding time +, blood smears show active phagocytosis of red cells, nucleated red cells and myeloblasts present. Animal thereafter killed with chloroform.

Post-mortem Findings.—One or two small hæmorrhages in skin.

Peritoneal Cavity.—No excess of fluid: no free blood: liver, enlarged, firm, and has mottled appearance with yellowish areas varying in diameter from 1 to 4 mm. Spleen enlarged and deep purple in colour.

Stomach and intestine both show some punctiform hæmorrhages. Kidney and suprarenals appear normal. Bladder distended with deeply blood-stained urine.

Pleural Cavity.—No gross change noted.

It will be seen that only in the case of the anti-platelet and anti-whole-blood sera did any appreciable drop in the platelet count occur, and that these two sera alone gave rise to purpura in the guinea-pig. In the case of the anti-whole-blood serum the number of hæmorrhages was much smaller than in the case of the anti-platelet serum. Both of these sera gave rise to hæmagglutination. There are several features of interest in the findings obtained with the former serum (anti-whole-blood). The intense destruction of red cells, with a corresponding drop in the number of platelets, is seen to occur side by side with a leucocytosis of 40,000. Blood smears made at this period showed active phagocytosis of red cells both by large mononuclear and polymorphonuclear leucocytes, particularly by the former cells, many of which were literally packed with red cells. The presence of many nucleated red cells, both normoblasts and megaloblasts, and of primitive white cells of the "lymphoidocyte" type, bore evidence of a reaction on the part of the bone marrow. Occasional cells of the latter type were seen undergoing mitotic division. Whether this leucocytosis was only part of the general bone-marrow reaction or due also to the call for phagocytes to remove the damaged red cells, it is difficult to say. The specificity of an anti-leucocyte serum when tested *in vivo* is well brought out by the experiment recorded in Table III. The platelets and red cells have fallen slightly in twenty-four hours, whereas the total leucocyte count shows a 75 per cent. reduction in

Two guinea-pigs were then taken. The one, B57, was inoculated intraperitoneally with the absorbed anti-platelet serum, the other with the diluted unabsorbed serum. Blood counts were made before inoculation, four and twenty-four hours after. The two animals were then killed and examined. The findings are recorded in Table VII.

TABLE VII.

Time of Observation.	Guinea-pig B57.			Guinea-pig B58 (Control).		
	Red Cells.	Plates.	Platelet /Red Cell Ratio.	Red Cells.	Plates.	Platelet /Red Cell Ratio.
*Before inoculation . . .	6,096,000	836,000	7.3	5,760,000	789,000	7.3
Five hours after . . .	5,712,000	111,000	49.3	5,536,000	36,000	153.6
Twenty-four hours after . .	3,648,000	35,000	102.4	3,920,000	55,000	70.6
<i>Post-mortem Findings.</i>						
Skin and sub-cut. tissue . .	Numerous hæmorrhages .			Numerous hæmorrhages.		
Lymph glands . . .	Deep red in colour . . .			As in B57.		
Peritoneal cavity . . .	Contains some free blood			Very little free blood.		
Stomach and intestines . .	Numerous hæmorrhages .			Numerous hæmorrhages.		
Spleen and liver . . .	Nil			Nil.		
Pleural cavity . . .	Contains a good quantity of free blood			No free blood.		
Lungs	Nil			Several large hæmorrhages.		
Heart	<i>Heart blood shows no agglutination</i>			<i>Heart blood shows well-marked agglutination.</i>		
Bleeding time in both animals lengthened considerably.						

* B57 received 4.0 c.c. "absorbed anti-platelet serum" (represents 1.0 c.c. undiluted serum) intraperitoneally.

B58 received 4.0 c.c. unabsorbed anti-platelet serum (diluted 1/4) intraperitoneally.

In both guinea-pigs the platelets have been almost entirely removed from the circulation, this diminution preceding the less extensive fall in the red count. No hæmagglutination was noted in the guinea-pig receiving the absorbed anti-platelet serum, whilst in the control animal this phenomenon was well developed. Both animals developed severe purpura. It was therefore obvious from this experiment that the red cell agglutinin present in anti-platelet serum played no part in the production of the hæmorrhages. What then are the factors concerned? Is the removal of the platelet from the circulation sufficient of itself to give rise to the hæmorrhages? It was a simple matter to test the correctness of this hypothesis, as it has been demonstrated by many workers (Aynaud (1911⁵), Roskam (1921⁶)) that the intravenous inoculation of such substances as peptone or Bordet's "anaphylatoxin" bring about a considerable though very transitory fall in the platelet count. The effect of "agar-serum" administered intravenously was therefore tried. The "agar-serum" was prepared by

absorbing fresh normal rabbit serum with 0.5 per cent. agar in normal saline (8.0 c.c. serum to 2.0 c.c. agar saline). This mixture was placed in the incubator at 37°C. for two hours and then allowed to stand at room temperature overnight, when the agar was removed by centrifugalisation. Small rabbits (800-1000 grms.) receiving 3.0-5.0 c.c. of this agar-serum intravenously showed a considerable though transitory fall in the platelet count. On no occasion was this accompanied by the production of hæmorrhages. The observations made on one of these rabbits are given in Table VIII.

TABLE VIII.

Time of Observation.	Red Cells.	Platelets.	Platelets /Red Cell Ratio.	Remarks.
*Before inoculation . . .	5,200,000	702,000	7.4	Some polypnoea after inoc. Soon recovered.
Thirty minutes after . . .	...	123,000	41.2	
One hour after	5,160,000	521,000	9.9	
One and a half hours after	Killed (chloroform)		and examined.	

* 3.0 c.c. "agar-serum" inoculated intravenously.

Post-mortem Findings.—Mesenteric vessels engorged: no hæmorrhages observed.

Since the removal of platelets from the circulation alone is unable to give rise to purpura, there must be additional factors concerned in the production of this condition. The mechanical factor (hæmagglutination) has been ruled out. No obvious change in the other blood elements has been brought to light which might help to explain the hæmorrhages, though Musser and Krumbhaar (1916⁷) consider the increased fragility of the red cell, which they find in experimental purpura, as being an essential factor. It is difficult to see why this should be. It has long been thought that some damage to the endothelium of the capillaries was essential to the production of purpura, and this is borne out by an examination of the tissues from guinea-pigs which have received inoculations of anti-platelet serum. The endothelium appears swollen and œdematous, the cells standing off the vessel wall. Supposing this to be the other factor concerned in the genesis of the hæmorrhage, then it should be supplied by any of the sera prepared against the blood elements, since they are all more or less closely related genetically to the endothelial cell. Assuming this to be correct, we then have two factors to consider, viz., the damage to the capillary endothelium and the removal of the platelet from the circulation. It has been already seen that anti-red-cell and anti-leucocyte sera are unable to give rise to purpura, presumably because the second factor is absent. It is in anti-platelet serum only that we find these two factors combined. If, however, an animal were inoculated first of all with an anti-red-cell serum and after a

suitable interval to allow for action on the endothelium of the vessels the platelets were removed, even temporarily, from the circulation, then we should have present in that animal the conditions necessary for the production of hæmorrhage and purpura should result.

MECHANISM OF THE HÆMORRHAGE.

To test this hypothesis the following experiment was carried out. A small rabbit (900 grms.) was inoculated with 0·75 c.c. anti-rabbit-red-cell serum intravenously. The red cells and the platelets were counted before and four and a half hours after inoculation. It then received 4·5 c.c. "agar-serum" intravenously, and the platelets and red cells were again counted after the lapse of thirty minutes. One hour after the second inoculation the animal was killed with chloroform and examined. *The post-mortem findings were similar to those produced by an anti-platelet serum.* The details of this experiment are summarised in Table IX.

TABLE IX.

Time of Observation.	Red Cells.	Platelets.	Platelet /Red Cell Ratio.	Remarks.
*Before inoculation .	4,800,000	631,000	7·6	Rabbit very collapsed. Breathing embarrassed. Passed some blood-stained urine.
3.0 p.m.	4,200,000	558,000	7·7	
†3.50 p.m.	3,680,000	92,000	39·9	
4.10 p.m.	Killed (chloroform)		and examined.	

* 0·75 c.c. anti-rabbit-red-cell serum inoculated intravenously 10.30 a.m.

† 4·5 c.c. "agar-serum" inoculated intravenously 3.10 p.m.

Post-mortem Findings.—Skin and subcutaneous tissues show a number of small hæmorrhages particularly in loose tissue over back.

Peritoneal Cavity contains some free, blood-stained fluid and one or two blood clots of moderate size: great omentum shows some small hæmorrhages: pancreas, one or two petechial hæmorrhages: intestine, numerous small punctiform hæmorrhages, particularly in lower portion of descending colon: kidneys and suprarenals, no macroscopic change: bladder, full of blood-stained urine: urine contains some unchanged red cells and granular casts: liver drips with blood on section—surface has a "mottled" appearance: spleen slightly enlarged and of deep purple colour.

Pleural Cavity.—Numerous hæmorrhages along the course of intercostal vessels: lungs show numerous hæmorrhages about 2 mm. in diameter.

This experiment was repeated with a precisely similar result. It has been shown that the "agar-serum" alone was unable to produce this pathological picture, and anti-red-cell sera, when tested in the guinea-pig, did not reduce the platelet count appreciably nor give rise to purpura. The effect of an anti-red-cell serum alone was further controlled by inoculating rabbits with one intravenously. This was done on two occasions and in neither case were any hæmorrhages produced. The details of one of these control experiments are given in Table X.

TABLE X.

Time of Observation.	Red Cells.	Platelets.	Platelet /Red Cell Ratio.	Remarks.
Before inoculation	4,240,000	588,000	7.2	
0.75 <i>Anti-rabbit-red-cell Serum inoculated intravenously.</i>				
Five and a half hours after	3,880,000	562,000	6.9	Animal somewhat collapsed after inoculation, but had recovered in thirty minutes.

Animal killed (chloroform) and examined.

Post-mortem Findings.—Skin and subcutaneous tissue: no hæmorrhages.

Peritoneal Cavity.—Vessels of mesentery engorged, no hæmorrhages: spleen, slightly enlarged, deep purple in colour: liver, kidney, and suprarenals appear unchanged: bladder contains blood-stained urine.

Pleural Cavity.—Nothing abnormal noted: no hæmorrhages.

Similarly no hæmorrhages are produced when the platelets are removed from the circulation of a rabbit which has received four and a half hours previously a normal foreign serum.

DISCUSSION.

It has been shown then that anti-platelet serum when inoculated into the animal for which it is specific produces purpura, whereas the sera prepared against the other blood elements—red cells, leucocytes, serum—do not. It is now well established that the hæmorrhagic diathesis in man is associated with a low platelet count, this tendency being the most severe in those cases where the platelets have almost entirely disappeared from the circulating blood. The destruction of the platelet or its removal from the circulating blood (for we are not certain of its fate) is brought about by anti-platelet serum. The other anti-sera do not produce this change. Obviously this is one of the factors concerned in the production of purpura. That it is not the only one is shown by the experiments with “agar-serum.” Although by this means the platelet counts can be very considerably diminished, hæmorrhages do not occur. In a recent paper by Roskam (already referred to), attention is drawn to the fact that in animals in which a reduction of the platelet count had been produced by the intravenous injection of a solution of gelatine, the bleeding time was only slightly prolonged. Now an increased bleeding time is a constant feature of purpura. Roskam therefore concludes that there must be some other change occurring in this condition, most probably some damage to the capillary endothelium. First proposed by Nolf, this view was also held by Ledingham, and a histological examination of the tissues from purpuric animals supports this contention, as change in the vascular endothelium, similar to those described by Findlay

(1921⁸) in experimental scurvy, can be made out, namely, a swollen and œdematous condition of these cells. It might be argued that this swelling of the capillary endothelium, with the consequent narrowing of the lumen of the vessel, would bring about a slowing of the circulating blood. The platelets would then adhere to the altered endothelial cells, leading eventually to a plugging of the vessel and its ultimate rupture. No evidence, however, has been found to support this view. Further, if a guinea-pig is given an inoculation of anti-platelet serum, and one to two hours later it is anæsthetised and its mesenteric vessels observed under the microscope, it is seen that the hæmorrhages form along the course of the vessels in which the circulatory current is not appreciably slowed. No evidence of plugging of the capillaries is to be seen, and, as far as one can see, the majority of the hæmorrhages appear to form gradually at different points along the course of the vessel, by what appears to be an exaggerated form of diapedesis. It would seem that in purpura, produced by means of an anti-platelet serum, the sequence of events in the production of hæmorrhages is as follows: the capillary endothelium is first of all damaged, and later, when the platelets have been greatly reduced in number in the circulating blood, a leakage of red cells through the capillary wall into the surrounding tissues takes place. Had the platelets been present hæmorrhages would not have occurred, because their presence in the outer more slowly moving stream, together with the leucocytes, would have prevented the rapidly moving central stream of red cells from coming in contact with the endothelium and of finding out its weak spots. They would also have helped in making good the deficiencies in the vessel wall caused by the endothelial damage.

CONCLUSIONS.

1. Of various anti-sera prepared by immunisation with blood elements (cellular and otherwise), anti-platelet serum alone produces purpura.

2. This purpura is the result of the action of platelet antibody alone and takes place independently of any hæmagglutination.

3. An extensive, though temporary, reduction in the number of platelets in the circulating blood of the rabbit does not give rise to purpura.

4. Experimental evidence has been brought forward to show that the two main factors concerned in the production of the hæmorrhages are:—

(a) Toxic action on the endothelium of the vessels.

(b) Removal of the platelets from the circulation.

5. The serological specificity of anti-leucocyte serum, as far as blood cells are concerned, is paralleled by its specificity when tested *in vivo*.

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