THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

II. The Effect of Pneumococcus Extract on the Blood Platelets and Corpuscles.

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It has been shown (1) that pneumococcus extracts produce purpuric lesions in white mice, rabbits, and guinea pigs. A number of toxic substances (benzene, saponin, diphtheria toxin, etc.) also have the property of producing purpura in experimental animals. It appears from previous studies that a combination of factors is involved in the production of experimental purpura. One of them is the excessive diminution in the number of blood platelets, which may be due either to their actual destruction, or damage to their seat of origin; the other is injury to the capillary walls. It seemed of interest, therefore, to study the variations in number of the blood platelets in white mice which were injected with pneumococcus extract.

Method Employed in Counting Platelets.—It was not possible to count the platelets by the direct method on account of the small quantities of blood available in mice. The indirect method was therefore adopted, in spite of the errors inherent in it, and was found satisfactory in that it gave a conception of the approximate number of blood platelets present.

The mouse's tail was warmed over an electric bulb to insure a free flow of blood. The tail was then laid on a block of wood with the tip immersed in normal salt solution containing 2 per cent sodium citrate. A short piece of the tail was cut off with a razor blade, and as the blood began to flow, the tail was removed from the citrate solution, and touched to a clean glass slide. The drop was spread over the surface with the edge of another slide. Four slides were made for each count. Haste to prevent clumping of the platelets was not necessary because of the presence of the anticoagulant. More blood was taken for erythrocyte and leucocyte counts and the bleeding was stopped by searing. The slides for the platelet counts were stained with Wright's stain in the usual manner. After

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the relative number of blood platelets and erythrocytes had been determined from the stained smears, and the actual number of erythrocytes had been found, the number of blood platelets was determined by proportion.

Care was exercised to prevent an unnecessary amount of bleeding. Control counts after similar repeated bleedings in normal mice showed no marked changes referable to the loss of blood.

EXPERIMENTAL.

The average number of erythrocytes in normal mice was found to be 8,000,000 and the platelets 1,800,000 per c.mm.

Effect on Platelets.—The mice were injected intraperitoneally with doses of 0.3 to 0.6 cc. of extracts obtained from Group IV, Type II, Type III, and the variant form of Type I pneumococcus. The method of preparation of these extracts has been described by Avery and Neill (12). All gave similar results. Counts were made at varying intervals, from half an hour to several days. Variation in the platelet count was observed in twenty mice.

A rapid fall in the number of platelets occurred soon after the injection of the extract. In one instance a third of the total number disappeared within 20 minutes. Usually the greatest diminution occurred after 24 hours. The lowest count obtained was about 8000 per c.mm. The number increased subsequently in the mice that survived the effects of the extract and reached normal in from 4 to 9 days. Thereafter there was an increase above normal lasting 2 to 12 days and by the 14th to the 20th day there was a return to normal which was maintained. In mice which failed to show purpura the platelets did not fall below 800,000 per c.mm. As a rule, purpura occurred only when their number was less than 500,000 per c.mm. Some specimen results are recorded in Tables I and II and illustrated by Graphs 1 and 2.

In general, the curves obtained by plotting the number of blood platelets in white mice, following the injection of pneumococcus extract, were similar to those obtained during the course of pneumonia, a number of other infectious diseases, and following the injection of certain toxic substances or antiplatelet serum in experimental animals. The initial reduction in the number of blood platelets was followed by a rise which exceeded the normal count, and later returned to normal.

In order to determine whether a cumulative effect on the platelets

could be obtained, one mouse was given five daily injections of small doses of the extract (0.05 cc. daily). During the course of these injec-

TABLE I.

Changes in Platelets and Erythrocytes Following Injection of Pneumococcus Extract.

Mouse III injected intraperitoneally with 0.4 cc. of Type II extract.

| Time of examination.			No. of platelets.	No. of erythrocytes.	Remarks.
Before injection.............			1,500,000	8,130,000	
20 min. after injection......			1,000,000		
1 hr.	"	"	700,000		
2 hrs.	"	"	510,000		Slight purpura in right ear.
4 "	"	"	500,000	5,240,000	Purpura in feet and tail also.
24 "	"	"	160,000	4,300,000	Purpura in feet, tail, and ears.
52 "	"	"	140,000	4,300,000	" " "
96 "	"	"	240,000	2,400,000	Purpura disappearing.
108 "	"	"	810,000	2,240,000	No purpura observed.
192 "	"	"	2,000,000	2,900,000	
9 days	"	"	2,800,000	4,100,000	
12 "	"	"	2,100,000	4,700,000	
18 "	"	"	1,600,000	7,300,000	

TABLE II.

Changes in Platelets and Erythrocytes Following Injections of Pneumococcus Extract.

Mouse VI injected with 0.4 cc. Group IV extract.

| Time of examination.			No. of platelets.	No. of erythrocytes.	Remarks.
Before injection.............			2,000,000	8,200,000	
90 min. after injection.......			1,400,000		
4 hrs.	"	"	640,000		Purpura in right ear.
24 "	"	"	74,000	5,800,000	Severe purpura in both ears, feet, and tail.
72 "	"	"	750,000	4,200,000	Purpura disappearing.
5 days	"	"	3,000,000	5,900,000	Slight trace of purpura left.
9 "	"	"	5,700,000	8,200,000	
14 "	"	"	3,700,000	6,500,000	
21 "	"	"	2,000,000	8,000,000	

tions there was a gradual diminution in the number of blood platelets to 750,000 per c.mm., after which the number began to return toward the normal, when the experiment was discontinued.

Camera lucida studies did not reveal the variations in size of the platelets which have been observed during the course of pneumonia in man (2).
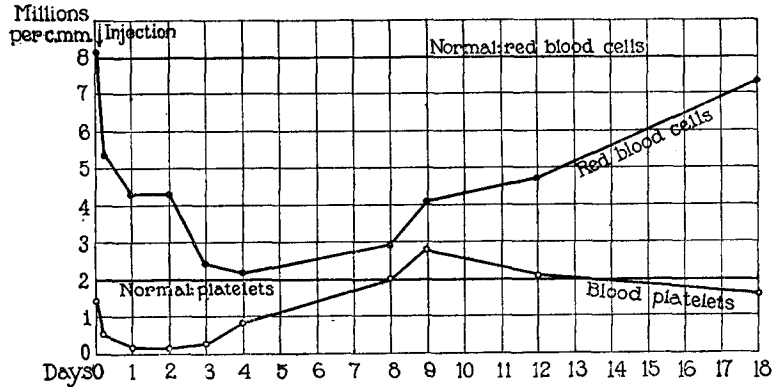
GRAPH 1. The effect of pneumococcus extract on the number of blood platelets and red cells. Graph illustrating Table I.

The mouse of 15 gm. was injected intraperitoneally with 0.4 cc. Type III extract.
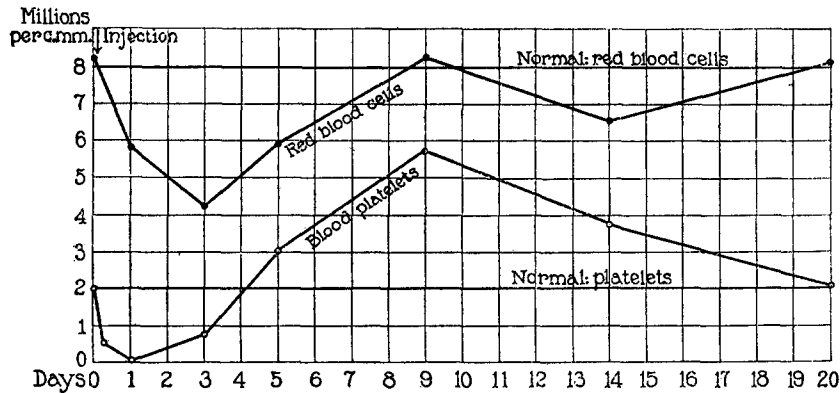
GRAPH 2. The effect of pneumococcus extract on the number of blood platelets and red cells. Graph illustrating Table II.

The mouse of 15 gm. was injected intraperitoneally with 0.4 cc. Group IV extract.

Effect on Erythrocytes.—Injections of pneumococcus extract caused a marked reduction in the number of erythrocytes as well as in the number of platelets as shown in Tables I and II. The rate of dim-

inution was somewhat slower for erythrocytes, but the diminution continued even after the blood platelets had begun to increase in number. The reduction of erythrocytes was most marked in from 3 to 7 days, and there was a return to normal in 10 to 20 days. The diminution of erythrocytes usually amounted to 50 per cent or more. That this anemia was not entirely referable to hemorrhage into the tissues is concluded from the fact that anemia may follow injections of pneumococcus extract in the absence of a visible purpura. Conversely, severe purpura may develop without the occurrence of a severe anemia as will be shown further on.

An occasional mouse showed a marked lipemia following severe anemia. This has also been observed by Bloor (3) and Horiuchi (4), in experimental animals in which anemia had been induced artificially.

Effect on Leucocytes.—These cells did not show marked fluctuation. Frequently a slight leucocytosis was observed immediately after the injection of the extract, followed by a slight leucopenia.

Observations on the Lytic Action of Pneumococcus Extract.

The rapid diminution in the number of platelets and erythrocytes in the injected mice suggested that the destruction might be due to direct lysis of the cells. Accordingly the lytic effect of the extract was tested *in vitro*.

Human and rabbit platelets were obtained by rapidly running 30 cc. of blood into 3 cc. of saline solution containing 5 per cent sodium citrate. The mixture was centrifuged slowly for 5 minutes to sediment the red and white blood cells. The supernatant fluid was separated and centrifuged at high speed to obtain the blood platelets. The blood platelets and erythrocytes were washed separately twice, and each sediment suspended in saline. In performing the lytic tests, 0.5 cc. quantities of extract, suspensions of platelets, and erythrocytes were used.

It was found that the extract was lytic for platelets and erythrocytes in dilutions as high as 1:100 when incubated with them for 1 hour at 37°C. However, the lytic action was completely prevented when both platelets and erythrocytes were suspended in serum instead of salt solution. Blood platelets which had been suspended in serum and stronger concentrations of the extract appeared blurred when stained and examined under the microscope. No lytic action against leucocytes was demonstrated *in vitro*.

Similar observations were made when bile, saponin, or sodium oleate were employed as lytic agents. The bile we used was lytic for platelets in dilutions of 1:600, the saponin in dilution of 1:200,000, and the sodium oleate in dilution of 1:100,000. In the same or somewhat greater concentrations, however, none of these agents was lytic for platelets when suspended in serum.

Observations were made *in vitro* on the effect of heat on the thrombolytic activity of pneumococcus extracts. The extract was heated at 55°C. for 10 minutes; and titrations of it were made before and after heating. The results are shown in Table III.

TABLE III.

The Effect of Heated and Unheated Pneumococcus Extracts on Platelets and Erythrocytes in Vitro.

Dilution of extract.	Unheated extract.				Extract heated 10 min. at 55°C.			
	Platelets suspended in		Red blood cells in		Platelets suspended in		Red blood cells in	
	Serum.	Salt solution.	Serum.	Salt solution.	Serum.	Salt solution.	Serum.	Salt solution.
1:1	−	+++	−	+++	−	−	−	−
1:10	−	++	−	++	−	−	−	−
1:50	−	+	−	+	−	−	−	−

+++ = complete lysis.
++ = incomplete lysis.
+ = slight lysis.
− = no lysis.

It will be seen that heat destroys the lytic activity *in vitro* of the extract for blood platelets and for red blood cells, as Cole (5) and Avery and Neill (6) have already shown. However, heat does not destroy the activity of the purpura-producing constituent. Heated extract when injected into white mice causes changes in number of blood platelets similar to those following the injection of unheated extract. But the number of red blood cells alters much less. Some figures are given in Table IV.

Adsorption Experiments.—Pneumococcus extract was brought in contact with blood platelets and erythrocytes to determine the possibility of a selective adsorption. The extract was mixed with one-third its volume of washed red blood cells which had been heated for

TABLE IV.

Changes in Platelets and Erythrocytes Following the Injection of Heated Pneumococcus Extract.

Mouse IV, injected with 0.4 cc. of extract which was heated for 20 minutes at 56°C.

Time of examination.	No. of platelets.	No. of erythrocytes.	Remarks.
Before injection..........	2,300,000	9,600,000	
1 day after injection......	450,000	9,000,000	Marked purpura in both ears.
2 days " "	710,000	9,100,000	" " " " "
5 " " "	1,500,000	9,200,000	

TABLE V.

The Effect of Adsorption of Pneumococcus Extracts on Platelets and Erythrocytes in Vitro.

Extract adsorbed with	Action on red cells.			Action on platelets.	
	Dilution of extract.			Dilution of extract.	
	1:1	1:5	1:10	1:1	1:5
Red cells............................	—	—	—	—	—
Platelets............................	++	+	—	+	+
White cells.........................	++	+	—	+	+
Kaolin.............................	+++	++	++	+++	+
Unadsorbed........................	+++	++	++	+++	+

TABLE VI.

Changes in Platelets and Erythrocytes Following the Injection of Adsorbed Pneumococcus Extract.

Mouse X, injected intraperitoneally with 0.5 cc. of extract after adsorption with erythrocytes.

Time of examination.	No. of platelets.	No. of erythrocytes.	Remarks.
Before injection................	2,000,000	8,400,000	
1 day after injection...........	170,000	8,800,000	Purpura after 18 hrs.
3 days " "	840,000	3,100,000	

10 minutes at 70°C. Specimens of the material were kept both in the ice chest and in the incubator for $\frac{1}{2}$ hour. The extract treated in this manner was no longer lytic for either erythrocytes or platelets. Extracts similarly treated with blood platelets showed a lesser diminution in hemolytic and thrombolytic titer. (See Table V.)

The extracts submitted to treatment of the sort described were still capable of producing purpura. Moreover, extracts from which the hemolysin had been adsorbed with red cells still produced marked anemia when injected into mice. Table VI shows these findings.

Mixture of the extract with erythrocytes at ice box temperature resulted in a binding of the lysin without hemolysis. The red cells upon which was fixed the lysin, when washed thoroughly and suspended in cold saline, hemolyzed completely when warmed to room temperature.

DISCUSSION.

On the whole, the behavior of the blood elements after the injection of pneumococcus extract is similar to the effects observed after the injection of other toxins.

Bunting (7) found that when saponin is given intravenously to rabbits a destruction of platelets and erythrocytes occurs and there is a lesser injury to the leucocytes. Saponin also causes destruction of the cells in the bone marrow and in particular it causes an injury to the capillary walls resulting in extensive hemorrhages. Bunting found that the changes in the number of platelets follows a curve independent of those of the other blood elements.

Duke (8, 9) showed that diphtheria toxin and benzene in large doses are poisonous to the bone marrow and cause a fall in the number of platelets and red cells. He thinks that the platelets themselves are also affected by these agents and that this factor contributes to the diminution in numbers. He believes that the rapidity of the changes can be accounted for by assuming that the platelets normally are short lived bodies.

Our results seem to corroborate these views inasmuch as the evidence indicates that the diminution in the number of platelets and erythrocytes is brought about by some action other than direct lysis alone. The lytic action is prevented *in vitro* by heating the extract and the heated extract still causes purpura. Moreover, lysis by unheated extracts is inhibited by serum. Adsorption of pneumococcus extract with blood platelets or red cells renders the thrombolysin inactive *in vitro*; but this procedure does not prevent the extract from producing purpura and thrombopenia in white mice. The lesions were as extensive as those caused by unabsorbed extract. If the diminution in the number of platelets were due to lysis alone, the number would return to normal in a much shorter time than is the case. In instances

such as those described by Richardson (10) and Bunting in which bleeding was done to lower the number of blood platelets in rabbits, the number returned to normal in 2 or 3 days. Duke (8) has shown that when platelets are almost completely removed from the circulation in dogs by defibrinating blood and reinjecting it, the number returns to normal in 3 to 5 days. However, if toxic agents which poison the platelet-forming organs are injected, the rate of regeneration is markedly retarded.

The rapid diminution in number of platelets after the injection of pneumococcus extract seems to point to a direct action on the platelets in the circulation, while the slow rate or regeneration points to a toxic effect of the extract on the formation elements in the bone marrow. A few mice did not show purpura until 2 or 3 days after the injection. There may have been a delayed action on the circulating platelets; or, possibly a toxic action on the marrow which prevented the normal formation of platelets, until there were so few left that purpura resulted. Students of purpura know that injury to the capillary walls occurs. Intact vessels will probably not permit leakage, no matter how low the platelet count may be. Degkwitz (11) contends that the normal function of platelets is to plug up interstices between capillary endothelial cells. Should this be the case then primary injury to capillary walls need not occur.

The pathology of the purpuric lesions is being studied and search is under way for changes in the hemopoietic centers and capillary walls. Avery and Neill (unpublished work) have determined that the activity of pneumococcus extracts *in vitro* may be destroyed by heat and oxidation, but the hemolysin may still function as an antigen. Our data add evidence to the complicated nature of the hemolytic activity of pneumococcus extract.

SUMMARY.

A study has been made of the variation in number of the blood platelets, and the red and white blood cells of white mice injected with pneumococcus extract. The blood platelets were greatly diminished after the injection, the greatest decrease usually occurring after 24 hours. Purpuric lesions usually developed when the number of blood platelets became less than 500,000 per c.mm. Regeneration of

the platelets was accomplished by the 4th to the 9th day but there was an overregeneration and the return to normal did not take place until 2 weeks had elapsed.

The red cells were also greatly reduced in number, but the rate of their destruction and regeneration was somewhat slower than that of the platelets. The leucocytes were slightly if at all influenced by the pneumococcus extract.

Pneumococcus extracts were shown to be thrombolytic and hemolytic. Heat destroyed the activity of both the lysins *in vitro*. Heated extract produced purpura in mice but did not cause a severe anemia. Extracts adsorbed with either blood platelets or red blood cells showed a marked diminution in their thrombolytic and hemolytic activity *in vitro*. Such extracts, however, produced purpura as well as severe anemia and thrombopenia in mice.

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