THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

X. Concerning the Supposed Regulatory Influence of the Spleen in the Formation and Destruction of Erythrocytes.

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In the older literature of the physiology of the spleen appear numerous references to differences in the composition of the blood of the splenic artery as contrasted with that of the splenic vein, and in the recent literature observations are made upon the occurrence of a specific hemolysin in extracts of splenic pulp. The possible bearing of these observations upon the various studies[1] of the spleen reported from this laboratory during the past two years led to a critical examination of the general literature and to certain experiments already described, in the hope that light might be thrown upon some of the difficult aspects of the general problem. A careful study showed that the observations in question are subject to a large experimental error which renders the finding of slight differences in the composition of arterial and venous blood of doubtful value,—a reason why the work has not heretofore been utilized in our reports. Indeed the work probably would have been entirely disregarded if Banti had not recently claimed, in support of his theory of the splenic origin of icterus, that the blood of the splenic vein normally contains more free hemoglobin than the blood of the general circulation. In order to test this and other points we have repeated experiments upon which the assumption that the spleen has a direct or indirect influence upon the red cells is based.

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Much of the early work on this subject is not only contradictory but was done before the development of the present exact methods of blood examination. Thus Virchow (1) found fewer red cells in the blood of the splenic vein than in that of the artery; while Malassez and Picard (2) and Emelianow (3) report the opposite. On the other hand, later investigators, Vulpius (4) and Paton, Gulland, and Fowler (5), have found no constant or noteworthy differences.

Considering the spleen as a possible leucoblastic organ, numerous early observers (6) found relatively more leucocytes, especially so called young forms, in the blood emerging from the spleen, than in that entering it. Tarchanoff and Swaen (7) and also Virchow (1) could not find any noteworthy difference, whereas Paton, Gulland, and Fowler (5) noted a constant diminution in the number of leucocytes in the splenic vein as compared with the general circulation. In this connection Bulgak (8), who describes an increase in leucocytes in the splenic vein, states that this is true of the venous blood of all parenchymatous organs. Freyer (9) concludes from his comparative counts that the spleen has nothing to do with blood formation.

The preceding studies refer of course to mature animals. It is accepted that in fetal life the spleen has the power of extensive blood formation, and several reports are at hand to show that the adult spleen may undergo, in the presence of injury to the bone marrow, a myeloid metaplasia (10); that is, that it can regain under pathological conditions its fetal function. Whether or not the spleen may exert this power of blood formation in the adult under normal conditions is doubtful, though still an open question.

Although the spleen certainly destroys red blood cells, as is evident from the presence in it of large cells, phagocytic for erythrocytes, which are increased under certain pathological circumstances, there still exists doubt whether the destruction by phagocytosis is the only method of red cell disintegration. It is stated also that the erythrocytes, in their passage through the spleen, are so acted upon by some unknown substance as to become more susceptible to hemolysis. This is the basis of Bottazzi's (11) hemocatatonistic theory, which has recently received support from Banti (12) and his colleague Furno (13). In the course of an investigation of hemolytic splenomegaly, they studied normal animals and those receiving hemolytic serum and came to the conclusion that free hemoglobin can be demonstrated in the blood of the splenic vein in normal animals as well as in animals receiving hemolytic serum. It was found at times in the blood of other vessels but in less amounts than in the splenic vein. They consider the findings as evidence of hemolysis in the spleen. The red blood cells of the splenic vein were found also to be less resistant to hypotonic salt solution than were those of the general circulation. Observations by Chalier and Charlet (14) on the resistance of red cells in the splenic artery and vein gave different results. Although the venous blood in general was slightly less resistant than arterial blood, this was reversed in the splenic system, so that the blood of the splenic vein was more resistant than that of the splenic artery and much more than the blood of other veins. Hammarsten is also said by Gabbi (15) to have found that the splenic vein blood was more resistant than the arterial. In the observations of Banti and Furno, the reference is to free hemoglobin in the serum and not to the increased hemoglobin content of venous or splenic blood described by several investigators (16).

The claim of Banti and Furno is surprising in that they state that the dissociated hemoglobin of the serum (*"emoglobin disciolta dal siero"*) is not only always present in the splenic vein of normal animals, but in sufficient quantities sometimes to be measured by a Sahli hemoglobinometer. It is to these observations that we have given especial attention in our work.

COMPARISON OF THE ARTERIAL AND VENOUS BLOOD.

Method.—From dogs under ether anesthesia, blood was obtained directly from the splenic artery and the splenic vein. Great care was exercised to disturb the vessels and the organ as little as possible, as it has been shown by Grigorescu (6) and Pribram (17) that the cell content of the blood may be greatly increased by congestion of the spleen. From a nick in the vessel wall of one of the branches of the artery or vein, fresh blood was drawn directly into Thoma blood-counting pipettes and the capillary tube of a von Fleischl hemoglobinometer. From another branch, blood was withdrawn by a syringe and immediately distributed to tubes containing different strengths of hypotonic salt solution designed to test the resistance of the red cells. Some of the blood was also set aside for similar tests with washed cells. For the determination of the presence of free hemoglobin in the serum, blood was collected in three ways: (1) in a paraffined centrifuge tube, (2) in a tube containing potassium oxalate, and (3) by drawing it directly into tubes through capillary points which were then sealed. All three samples were then centrifuged and the serum was examined for hemoglobin by visual inspection and the spectroscope. Smears for differential counts were made at times from the blood flowing directly from the vessel and at times from a drop from the syringe. Finally, tests for reticulated or skeined (young) red blood cells were made. This was done by letting a few drops of blood fall into a solution of brilliant cresyl blue, and, after standing fifteen or twenty minutes, the skeined forms in proportion to the unskeined or mature forms were counted in fresh smears. For the purpose of controls, blood from the femoral vein, and from the capillary circulation by puncture of the skin was occasionally collected.

Results.—The figures for the red and white cells, differential counts, and total hemoglobin in a series of five dogs show that as far as these estimations are concerned the blood of the splenic vein does

not differ greatly from that of the artery. The variations are not uniformly on one side and are all within the limit of error inherent in the methods of blood examination.

It is of interest that in these and other dogs, the red cells of the vein, in six of eight animals, showed more or less marked anisocytosis and inequality of staining, which were not seen to the same degree in the blood of the artery. Polychromatophilia was about equal in artery and vein. In two of the eight animals a few normoblasts were found in the splenic vein blood only. Control smears from the femoral vein of four dogs showed changes in the red cells about equal to that of the splenic vein, indicating that these changes are characteristic of venous blood in general rather than a specific change caused by passage through the spleen.

In regard to the presence of free hemoglobin in the serum, if we had depended on one tube only, we should have occasionally found apparent hemoglobinemia, both of the general circulation and of the splenic vein; but as in every set of three tubes, in a series of seven dogs, at least one was free of hemoglobin, we cannot support the view that free hemoglobin in demonstrable amounts is present normally either in the splenic vein or in the general circulation of the dog. Our experience forces us to the conclusion that the findings of other investigators are due to hemolysis after collection or are dependent upon the method of separating the serum.

As regards the resistance of the red cells, of which comparative tests were made on eight dogs, in five no difference was found between artery and vein; in the other three, the venous corpuscles were slightly less resistant. Two control tests with cells from the femoral vein showed these to have the same resistance as those of the splenic vein.

In seven comparative tests for skeined or reticulated red corpuscles, these were found five times to be more abundant in the splenic vein and twice more numerous in the artery; the differences were never very striking. Five controls from the femoral vein corresponded more closely to the splenic artery counts than those of the splenic vein.

Conclusions.—As a result of the various observations we conclude that the slight differences between the arterial and venous

blood of the spleen are within the limits of error inherent in the methods of blood examination and are not to be explained by a peculiar action of the spleen. In some instances peculiarities shown by the splenic venous blood are common to the venous blood of the general circulation. Banti and Furno's observation concerning the presence of free hemoglobin in the blood of the splenic vein is not confirmed.

THE HEMOLYTIC POWER OF SPLENIC EXTRACTS.

The histological evidence of the destruction of erythrocytes by phagocytic cells of the spleen has naturally suggested the possibility of the liberation by these cells of a ferment capable of acting extracellularly. If such a free hemolysin is present in the spleen it should be demonstrable in extracts of the spleen, and during the past few years several investigators have therefore tested the influence of such extracts upon red cells. The methods employed, based on the technique of Korschun and Morgenroth (18), are similar, but the results obtained have been contradictory.

Korschun and Morgenroth found in several organs a hemolytic substance of unknown origin, coctostabile and soluble in alcohol, which did not arise from constituents of the blood serum and was in no way peculiar to the spleen. Nolf (19), on the other hand, found that the hemolytic power of splenic extract was distinctly greater than that of the liver, mesenteric lymph nodes, or kidneys, but only slightly more than that of the lung. This hemolytic substance was specific for the species and was destroyed at 100° C. Achard, Foix, and Salin (20), repeating these experiments, showed that the final solution was strongly acid, presumably as the result of bacterial action, and that control tests made with precaution as to asepsis were uniformly negative. Widal, Abrami, and Brulé (21) in similar experiments could get no hemolysis with fresh extracts used on the day they were prepared; sometimes, also, extracts twenty-four to forty-eight hours old were without effect. From these results they conclude that the hemolytic substance is not a true hemolysin, but the product of cell autolysis. Iscovesco and Zacchiri (22) had previously shown that after placing the mixture of pulp and saline solution in the thermostat for fifteen to twenty hours, the filtered extract, on the addition of red cells and after standing two and one half hours in the thermostat, showed 2.5 to 8 per cent. hemolysis, as determined by the Dubosc colorimeter, and they conclude that the hemolytic power of splenic extracts is unimportant. Weill (23) found a weakly hemolytic substance in extract of spleen that was inactivated at 56° C. and reactivated with guinea pig serum. This was more powerful than a lymph node extract prepared in the same way, but much less powerful than the extract obtained from the spleen by long maceration. The latter was not destroyed below 80° C., and its action was hindered by adding

fresh serum. Extracts from lymph nodes prepared in the same way showed only slight hemolytic action and those from other organs were negative. Banti (12) and Furno (13) state that fresh extracts of the normal spleen sometimes have no hemolytic action, and sometimes a weak action which is increased on standing twenty-four to forty-eight hours on ice and is not destroyed by heating to 60° or even to 100° C. They consider it a cytohemolysin, normally present in the spleen in small amounts and much increased after the administration of hemolytic agents. Thus we find that Nolf, Weill, Banti, and Furno find splenic extracts to have a hemolytic action greater than that of other organs. Achard, Foix, and Salin, and Widal, Abrami, and Brulé, on the other hand, fail to find any hemolytic action of the fresh extract, and think it is found only after autolysis or bacterial decomposition of the spleen.

Our experiments were made with extracts from the spleens of three dogs. The technique described by Nolf was followed in the main with several additions in the way of control experiments. On washing through the aorta, it was found that the technique which will give a blood-free kidney or liver will not render the spleen bloodless. Various expedients were tried, therefore, to secure a hemoglobin-free extract. It was found that if the spleen, after washing through the aorta, was cut in small pieces and pounded with a pestle against a wire-meshed sieve placed in a mortar, with the aid of frequent washings with salt solution, a blood-free white mass was obtained consisting partly of reticulum and partly of adherent splenic pulp (extract A, table I). As it was possible that the hemolytic substance might not be retained, or in only small amounts, in this fraction, extracts were also made from that part of the spleen that was mashed through the sieve. This residue was of course distinctly blood-tinged, so that it was necessary, in order to remove the blood, to mix it with distilled water, centrifuge, discard the supernatant fluid, and repeat the process until colorless tissues were obtained (extract B, table I). In each case the material thus obtained was mixed with double the amount of salt solution and placed in the refrigerator. Tests were always made with extracts one or two hours old, a small portion being filtered off for this purpose, and in two instances also after eighteen and twenty-four hours. Control tests were made in one experiment with extracts of liver and mesenteric lymph nodes. As it was possible to wash the latter free of blood before removal from the body, an extract was easily obtained by grinding the tissue in sand with mortar

TABLE I.

Character of extract.	Amount of splenic extract in c.c.								Salt solution control.	Distilled water control.
	1.95	1.5	1.0	0.5	0.3	0.2	0.1	0.05		
1. Dog 1. Fresh spleen extract A.....	—	V.S.	0	0	0	0	0	0	0	C.
2. Dog 2. Fresh spleen extract A.....	—	0	0	0	0	?	0	0	0	C.
3. Same. Extract B.....	—	0	0	0	0	0	0	0	0	C.
4. Same. After extraction in ice chest for 24 hrs.....	—	V.S.	V.S.	0	0	0	0	0	0	C.
5. Dog 3. Fresh spleen extract A.....	V.S.	0	0	0	—	0	0	0	0	C.
6. Spleen extract A after extraction in ice chest for 24 hrs.....	—	0	0	0	0	0	0	0	0	C.
7. Fresh spleen extract (boiled).....	0	0	0	0	—	0	0	0	0	C.
8. Fresh liver extract.....	0	0	0	V.S.	—	0	0	0	0	C.
9. Fresh mesenteric lymph node extract.....	0	0	0	0	—	V.S.	0	0	0	C.
10. Mesenteric lymph node extract after extraction in ice chest for 24 hrs.....	—	0	0	?	V.S.	M.	V.S.	0	0	C.

0 = no hemolysis; ? = doubtful hemolysis; V.S. = very slight hemolysis; M. = marked hemolysis; C. = complete hemolysis; — = no test.

and pestle and placing it as before in the ice chest with double the amount of salt solution. In two experiments the tests were made on the corpuscles of the animal furnishing the spleen; in one the corpuscles of another dog were used without a difference in result. The preparation of the washed red blood corpuscles, the dilutions, incubation, and so forth, were made according to Nolf's technique. Each tube contained 0.1 of a cubic centimeter of washed dog's corpuscles with varying amounts of splenic extract made up to two cubic centimeters with normal salt solution. Controls were made with normal salt solution and distilled water. The results are presented in table I.

Conclusions.—Fresh extracts of spleen are devoid of definite hemolytic action. Occasional trivial and irregular results, not to be explained, are found, but these occur likewise in the control extracts of liver and mesenteric lymph nodes. Extracts twenty-four hours old, prepared at low temperature, show little or no increase in hemolytic activity. Boiled splenic tissue, extracted in the cold for twenty-four hours, is inert.

THE INFLUENCE OF THE INTRAPERITONEAL INJECTION OF SPLENIC EXTRACT.

The changes in the blood picture following experimental removal of the normal spleen suggest that changes of interest might be produced by a converse procedure; namely, introduction into the body of the products of splenic activity in the form of splenic extract. If temporary anemia follows removal of the spleen, one might expect that some temporary rise in the red cell count might follow the injection of splenic extract.

The literature concerning the spleen contains very few reports on this subject. Danilewsky (24) found a surprising increase in hemoglobin and red blood cells after a single subcutaneous or intraperitoneal injection of extract of spleen. This increase reached its height in from three to seven days and continued as long as the experiment lasted, usually eight days. In dogs with a dietary anemia, splenic extract caused an even greater rise; for example, of 40 per cent. hemoglobin and almost 2,000,000 red cells. Danilewsky assumed that his results were due to a stimulation of the bone marrow. This influence of the splenic extract was not destroyed by heating.

Danilewsky's work, however, is uncontrolled by injection of other organ extracts, and the rise noted extended over a surprisingly long period of time. Silvestri (25) records a single observation in which a dog, presumably dying from anemia, was apparently saved by the injection of splenic extract. In this connection it must also be noted that the clinical literature of this subject contains several reports (26) of the use, with good results, of extracts of spleen and bone marrow in the treatment of anemia.

Method.—We have tested the effect of splenic extract on four dogs, using as controls extracts of other organs similarly prepared and extracts of erythrocytes.

The usual examinations of the blood were made, and also determinations of the resistance of the erythrocytes to hypotonic salt solution and the percentage of skeined cells. As a rule two counts were made before injection, and daily counts after the injection until the blood picture had returned to normal, usually a period of from three to four days. Extracts were prepared from organs removed aseptically from dogs bled to death under ether anesthesia. The finely chopped organ was ground in a sterile mortar to a homogeneous pulp and extracted with double the volume of salt solution for two hours in the ice chest. Ten cubic centimeters of the filtered extract were injected intraperitoneally into dogs of about the same

weight. Defibrinated blood diluted 1 to 20 with normal salt solution was used in ten cubic centimeter amounts to control the possibility of the rise in red cell count being due to the influence of some constituent of the red cells. In no case did peritonitis or other infection result. The result of one of these experiments is shown in table II.

TABLE II.

Date (1914).	Hemoglobin.	Red blood cells.
Feb. 6	102	5,250,000
Feb. 7	101	5,650,000
	(10 c.c. splenic extract No. 16 injected.)	
Feb. 8	110	6,500,000
	(15 c.c. of same extract injected.)	
Feb. 9	110	7,040,000
Feb. 10	105	6,800,000
Feb. 11	96	5,330,000
Feb. 12	95	5,290,000
	(15 c.c. splenic extract No. 88 injected.)	
Feb. 13	101	5,700,000
	(10 c.c. of same extract injected.)	
Feb. 14	104	6,880,000
Feb. 15	98	5,860,000
Feb. 16	96	5,120,000
Feb. 19	106	5,540,000

This experiment shows that intraperitoneal injection of splenic extract causes a sharp rise in hemoglobin and red cell count, lasting only one or two days. This rise is repeated on reinjection of either the same or another splenic extract.

In each of three other experiments with splenic extract an increase in the number of red cells was obtained, but this increase was not always as marked as in the experiment presented; it was nevertheless always greater than that caused by the use of control extracts of liver, kidneys, or blood.

The study of the resistance of the red cells may be dismissed with the statement that no noteworthy differences were found after injection of any extract. The skeined cells also showed no constant change. We had hoped that as the latter are supposed to be young forms of erythrocytes, they would be found to be increased after the injection of splenic extract had caused a rise in the red cell

count. Only once, however, when the percentage rose from 0.5 to 2 per cent., was this noticed. On the other hand, in two experiments they were not found at all in the blood after injection.

Intraperitoneal injection of splenic extract is usually followed by an increase in the total number of leucocytes, consisting chiefly of the polymorphonuclear forms. A similar rise occurred in one of three injections of liver and kidney, and in one of two of defibrinated blood. Several grades of transitional cells appeared in increased numbers. Eosinophils were present in increased numbers in two of the four dogs receiving splenic extracts, but were also definitely increased in two of the five controls.

Conclusions.—Intraperitoneal injection of saline extracts of fresh spleen constantly causes a sharp increase in red cell count and hemoglobin content. The rise is evanescent, lasting but one or two days, and may be followed by an equally evanescent drop below normal. Similarly prepared extracts from other organs fail to give this rise. No noteworthy change is found in the resistance of the red blood cells to hypotonic salt solutions, or in the number of skeined or reticulated erythrocytes, after the injections of the various organ extracts.

A temporary increase of polymorphonuclear and transitional leucocytes usually follows the use of spleen extract, but may occur also, though less frequently, after the injection of liver and kidney.

The constant increase of red cells in the peripheral circulation after injection of spleen, in view of the tendency to anemia following splenectomy, suggests that the spleen normally may exert a stimulating effect upon the formation of red cells in the bone marrow.

THE INFLUENCE OF FEEDING SPLEEN TO SPLENECTOMIZED DOGS.

This study complements that just described in that spleen in large amounts was fed to splenectomized animals. The object was to determine whether through the influence of a possible internal secretion of the spleen the anemia following splenectomy might be prevented. The procedure is of course analogous to thyroid feeding in insufficiency of the thyroid gland, and has an advantage over the injection of extracts in that it may be continued over long periods of

time without the possibility of the complications occasionally occurring after injection. These experiments, it was hoped, would show whether or not the spleen exerts some effect upon the hemopoietic system through peculiar bodies concerned perhaps in an internal secretion. Thus, if the anemia following splenectomy depends upon the absence of a normal stimulus to the hemopoietic system in general, or to some part of it, as the bone marrow, furnished normally by the spleen, the feeding of normal fresh spleen unmodified by heat or chemicals would supply this secretion and there would be no anemia after the removal of the spleen.

Method.—Five dogs were used. Four of these were given a diet consisting of raw hashed beef spleen, lard, and cracker meal in amounts estimated, according to the weight of each animal, to suit its caloric needs. Of these, three were splenectomized and one served as a control. As an added control, a splenectomized dog received a diet in which casein was substituted for beef spleen. The red cells and the hemoglobin were estimated several times before splenectomy and afterwards counted twice a week for three weeks. After this they were counted every week for approximately five weeks. No preliminary counts were made until a dog had been on the special diet for at least a week, and splenectomy was not performed until two weeks later.

Results.—Of the three splenectomized dogs receiving spleen in the diet, one showed a very slight decrease in red cells and hemoglobin, but the other two developed the usual anemia of splenectomy. Thus one with an initial red cell count of 6,200,000 showed on the 12th day 4,710,000 red cells with return on the 54th day to 6,040,000. This animal received daily 150 grams of beef spleen. The other dog receiving daily 275 grams of spleen showed a change in red cell content of about the same degree. In the splenectomized dog not fed spleen, the red cells fell from 5,550,000 to 4,210,000 on the 19th day with return to 5,060,000 on the 54th day. In this dog the hemoglobinemia reached its lowest level (65 per cent.) on the 12th day, and remained at about that point until the 26th day. In neither of the other splenectomized animals receiving spleen did the hemoglobin fall below 75 per cent. The normal dog, receiving 150 grams of spleen daily, showed no change in the blood.

It is evident that in two dogs, despite the feeding of spleen, an anemia was produced that ran a course similar to that which we have previously shown to be the rule in splenectomized dogs (27). In view of these definite results, the absence of marked anemia in the third splenectomized dog must be considered as the result of factors other than the feeding of spleen.

Incidentally it was found that the resistance of the red cells to hypotonic salt solution was increased in all splenectomized dogs, thus confirming the work of Karsner and Pearce (28).

Conclusion.—The feeding of fresh raw spleen to splenectomized dogs has no clearly defined influence in preventing the anemia which usually occurs after splenectomy.

GENERAL SUMMARY.

1. The blood of the splenic artery and vein shows either no differences, or only such slight irregular variations as may be due to the errors inherent in hematologic methods, or are common to arterial and venous blood of the general circulation.

2. The observation of Banti and Furno that free hemoglobin occurs in the blood of the splenic vein is not confirmed.

3. Extracts of the spleen have no definite hemolytic action *in vitro*.

4. Intraperitoneal injection of fresh saline extracts of the spleen causes in the dog a sharp increase in the number of red cells and the hemoglobin content which lasts for one or two days and may recur on a second injection. Extracts of liver, kidney, and erythrocytes similarly prepared do not give this effect. This observation supports Danilewsky's theory that the spleen may exert a stimulating effect upon the formation of red cells in the bone marrow.

5. On the other hand, the feeding of raw beef spleen to splenectomized dogs over long periods of time has no clearly defined influence in preventing the anemia which usually follows splenectomy.

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