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

IV. A STUDY, BY THE METHODS OF IMMUNOLOGY, OF THE INCREASED RESISTANCE OF THE RED BLOOD CORPUSCLES AFTER SPLENECTOMY.*

BY HOWARD T. KARSNER, M.D., AND RICHARD M. PEARCE, M.D.

(From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.)

In the first and third communications of this series it was shown that a dog that has been splenectomized for some time is more resistant to a specific hemolytic immune serum than is the non-splenectomized dog, and that to produce hemoglobinemia and hemoglobinuria, or jaundice, in such an animal it is necessary to administer larger amounts of serum than in the normal animal. Tests in vitro with hypotonic salt solution and with hemolytic serum showed that this diminished susceptibility of the animals to the action of a hemolytic serum was in all probability due to an increased resistance of the red cells. These tests, however, were made irregularly and were but incidental to other phases of the general investigation. In the present communication the results of a special study of this problem by the accurate and detailed methods of immunology are presented in detail.

Increase in resistance of the red cells to hemolytic agents after splenectomy is not a new observation. It has been noted in the dog by Bottazzi,* Banti,* Pearce, R. M., Austin, J. H., and Eisenbrey, A. B., I. Reactions to Hemolytic Serum at Various Intervals after Splenectomy, Jour. Exper. Med., 1912, xvi, 363; Pearce, R. M., Austin, J. H., and Musser, J. H., Jr., II. The Changes in the Blood Following Splenectomy and Their Relation to the Production of Hemolytic Jaundice, ibid., 758.

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Blood Destruction and Regeneration.

Vast, Pugliese and Luzzatti, and Joannovics, in the guinea pig by Gabbi, and in the rabbit by Domenicci. The hemolytic agent used by these investigators was, as a rule, tolulylendiamine, though occasionally pyridin (Pugliese and Luzzatti), acetylphenylhydrazine (Banti), and specific hemolytic immune serum (Joannovics) have been employed. In general it has been assumed that the increased resistance to the toxic influence of the drug, or the failure to produce hemoglobinemia or jaundice, is due to increased resistance of the red cells. Banti invokes also the absence of the alleged hemolytic function of the spleen, and Vast also believes that for the destruction of red cells the intervention of the spleen is necessary. This point of view is to some extent supported by Joannovics. Pugliese and Luzzatti, on the other hand, deny the influence of the absence of the spleen and point to the presence, after repeated doses of pyridin, of newly formed immature nucleated corpuscles, poor in hemoglobin, which they think may possibly be more resistant to pyridin than are the normal cells. Few exact studies of the condition of the corpuscles have been made, and these are limited, as in the investigations of Brissaud and Bauer, Chalier and Charlet, and Pel, to the use of hypotonic salt solutions. No careful study of the resistance of these cells to hemolytic serum in vitro has been made, and the allied problems of possible antihemolytic action of the serum and of possible changes in complement content have not been investigated.

Brissaud and Bauer, working with two rabbits and using hypotonic salt solution of varying strengths, found a decreased resistance of the red cells during eight to ten days after splenectomy with, "after the lapse of time," a return to normal, but no increase in resistance. They do not, however, state how long the experiment was continued. Chalier and Charlet, using both the rabbit and the dog, and the salt solution test, conclude that splenectomy is followed apparently by a slight increase in the resistance of the red cells. Pel, in a very extensive study, found that as an average of fifty-eight determinations on normal dogs the first trace of hemolysis occurred in 0.42 per cent. salt solution as compared with 0.35 as the average for thirty observations on splenectomized dogs; the average concentration at which hemolysis was complete was, for normal dogs, 0.30 per cent. salt solution, and for splenectomized dogs 0.23 per cent.

\* Vast, cited by Joannovics, loc. cit.; original not available.
\* Pugliese, A., and Luzzatti, T., Contribution à la physiologie de la rate, Arch. ital. de biol., 1900, xxxiii, 349.
\* Domenicci, cited by Pel, loc. cit.; original not available.
\* Brissaud and Bauer, Recherches sur la résistance des globules rouges chez le lapin, Compt. rend. Soc. d. biol., 1907, lxii, 1068.
\* Chalier, J., and Charlet, L., État de la résistance globulaire chez l'animal normal et splénoctomisé, Jour. de physiol. et de path. gén., 1911, xiii, 728.
Thus in both series of observations the increased resistance of the splenectomized animals was the equivalent of 0.07 per cent. salt solution. The difference may be expressed in another way; in the fifty-eight observations on normal animals hemolysis began in all but one test in solutions of 0.48 to 0.40 per cent., while of thirty observations on splenectomized animals, in all but two it began in solutions of 0.38 to 0.30 per cent.; likewise complete hemolysis occurred in the normal group only seven times below 0.30, while in the splenectomized group it occurred always below 0.30 per cent. solution. The resistance of the red cells was found to increase gradually and reach its maximum after about two months; further lapse of time showed no tendency to return to normal. The longest period after splenectomy was two years and four months.

Pel makes a general statement concerning the influence of the serum to the effect that the serum of a splenectomized dog added to the red cells of a normal dog does not increase the resistance of the latter to hypotonic salt solution, and vice versa, that the addition of normal serum to the red cells of a splenectomized dog does not decrease their resistance.

Blood counts showed a slight decrease in the number of red cells after splenectomy but not enough in the opinion of Pel, in view also of only slight changes in the percentage of hemoglobin, to have any relation to the increased resistance of the red cells. As to the factors responsible for the increased resistance, Pel offers no explanation.

EXPERIMENTAL PART.

In the present investigation we have used six dogs. One had been splenectomized ten days, a second, thirty days, and a third, four months before. The last animal had been given hemolytic immune serum two months before the beginning of the immunological tests, and therefore as a control for this animal, a dog which had received hemolytic immune serum five weeks previously, but was otherwise normal, was included in the series, and as controls for the other two animals two normal dogs were used.

For the determination of the resistance of the corpuscles to hypotonic salt solution we employed, with slight modifications, the method of Theobald Smith, as used by Gay. Chemically pure sodium chloride was dried for two hours at 170° C. and immediately weighed in amounts necessary to make 500 cubic centimeter volumes of salt solution ranging from 0.1 to 0.5 per cent. in steps of 0.025 per cent. In order to be sure of approximately the same

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# TABLE I

<table>
<thead>
<tr>
<th>Corpses of</th>
<th>Percentage strength of salt solutions (June 10, 1912)</th>
<th>0.075</th>
<th>0.200</th>
<th>0.250</th>
<th>0.300</th>
<th>0.350</th>
<th>0.400</th>
<th>0.450</th>
<th>0.500</th>
<th>0.550</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 30: Normal</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>40</td>
<td>Trace</td>
<td>0</td>
</tr>
<tr>
<td>Dog 33: Normal</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>40</td>
<td>20</td>
<td>Trace</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dog 31: Splenectomy, May 31</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>20</td>
<td>Trace</td>
<td>Trace</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dog 46: Splenectomy, May 10</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>40</td>
<td>20</td>
<td>Trace</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dog 44: Splenectomy, Feb. 10, Immune serum, April 16</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>40</td>
<td>20</td>
<td>Trace</td>
<td>Trace</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dog 43: Immune serum, April 21</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>20</td>
<td>Trace</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
volume of corpuscles in the anemic as in the normal bloods, the
gently defibrinated blood was centrifuged and the serum drawn off.
0.1 of a cubic centimeter of the corpuscular mass was measured
accurately in a graduated pipette and placed in three cubic centi-
meters of each of the various salt solutions. Standard colorimetric
scales for comparison were made by laking red cells with distilled
water; thus the laking of 0.4 of a cubic centimeter of the corpuscu-
lar mass in twelve cubic centimeters of distilled water represented
a standard of 100 per cent. hemolysis. Dilutions of this solution
were made so as to have tubes showing the color values of 80, 60, 40,
and 20 per cent. hemolysis. Less than 20 per cent. hemolysis is in-
dicated in table I as a trace of hemolysis. In most instances this
scale was entirely satisfactory, but occasionally, although a tube
showed 100 per cent. hemolysis colorimetrically, there was, on shak-
ing, a slight macroscopic sediment of incompletely hemolyzed cor-
puscles; this result is indicated by a minus sign after the approx-
imate percentage of hemolysis. Upon adding the corpuscles to
the salt solution, a preliminary reading was made and the mixtures
were placed in the refrigerator. The final reading at the end of
eighteen hours was made the basis for table I.

It will be seen that the blood of the normal dogs (30 and 53)
shows hemolysis in fairly high percentages of salt solution, but
that the resistance is increased in all the abnormal animals. It is
true that dog 46, a splenectomized animal, shows initial hemolysis in
the same percentage of salt solution as normal dog 53; but inspec-
tion will show that whereas in the normal dog hemolysis is com-
plete at 0.350 per cent., it is not complete in dog 46 until 0.300 per
cent. is reached. There can be no doubt that the cells of dog 24,
the animal which had been longest splenectomized, show the greatest
degree of resistance. That this resistance is due for the most part,
if not entirely, to splenectomy, is, in view of results with bloods 30
and 53, most probable. On the other hand, it is evident, as shown
by the experience with blood 43, that the administration of a hem-
olytic immune serum is followed by an increased resistance of the
red cells in the absence of splenectomy.

In order to determine the resistance of the corpuscles to a specific
hemolytic immune serum the following technique was employed. The corpuscles were washed three times in 0.85 per cent. salt solution, and blood suspensions were made of 5 per cent. of red cells as contained in the centrifuged corpuscular mass. The latter rather than whole blood was used because the use of whole blood would be fallacious in the case of anemic animals. The immune serum was titrated against normal corpuscles, guinea pig complement was used in doses of 0.1 of a cubic centimeter, and the experiment arranged as indicated in table II.

**TABLE II.**

<table>
<thead>
<tr>
<th>Dilutions of immune serum. (June 14, 1912)</th>
<th>x/50</th>
<th>x/100</th>
<th>x/150</th>
<th>x/200</th>
<th>x/250</th>
<th>x/300</th>
<th>Complement control</th>
<th>Ambrocegum control</th>
<th>Blood control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 30. Normal...</td>
<td>C.H.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 53. Normal...</td>
<td>C.H.</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 57. Splenectomy, May 31...</td>
<td>P.H.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 46. Splenectomy, May 19...</td>
<td>P.H.</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Dog 44. Splenectomy, Feb. 10...</td>
<td>P.H.</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 43. Immune serum, April 16...</td>
<td>P.H.</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 43. Immune serum, April 21...</td>
<td>P.H.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Technical limitations prevent, in this experiment, as close an estimation of resistance as is possible with hypotonic salt solution, but it can be seen readily that whereas dilutions of 1/20 and 1/50 produced complete hemolysis of normal corpuscles, the corpuscles of the abnormal animals were resistant to these dilutions. The fact that the corpuscles of dog 24, which had been splenectomized four months previously, were most resistant is shown by the fact that whereas partial hemolysis appeared in all other corpuscles in dilutions of 1/250 and 1/300, the corpuscles of this dog resisted completely hemolysis at such dilutions. Here again the results with the blood of dog 43 demonstrate that the administration of an hemolytic

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*Prepared by injecting the rabbit five times with 5 c.c. of dog blood, at intervals of five to seven days.

*In the tables, C.H. = complete hemolysis; H. = hemolysis; P.H. = partial hemolysis.
serum increases the resistance of the red cells, irrespective of splenectomy.

Since it has been shown by Karsner and Pearce\textsuperscript{16} that agglutination limits hemolysis, it was considered wise to avoid possible error due to such action. The agglutinative effect of the immune serum was therefore examined in the same dilutions as were used for hemolysis. It was found that the agglutinability of the corpuscles was the same for all bloods except that of dog 43, the corpuscles of which formed somewhat smaller and less firm clumps than the others, but agglutinated at the same time and in the same dilutions as the rest. It was thus conclusively shown that the variations in resistance to hemolysis do not depend on variability of agglutination.

The fact that washed corpuscles were used in the experiments with immune serum would indicate that the increase in resistance is indeed a property of the corpuscles themselves, but in order to prove this point absolutely, the sera of all six dogs were examined for antihemolytic properties. It has been shown by Karsner and Pearce\textsuperscript{17} that fresh dog serum has a definite antihemolytic property in an homologous hemolytic system. In the experiments designed to determine whether this property is augmented after splenectomy, an immune serum was carefully titrated with a constant dose of guinea pig complement. Two rows of tubes were used; in the tubes of one row were placed 0.5 of a cubic centimeter of a dilution of dog serum, as indicated in table III, and one dose (0.5 of a cubic centimeter) of amboceptor. In the second row the same amount of dog serum and two doses of amboceptor were placed. The tubes were then incubated for one half hour, and to the first row one dose of complement was added, to the second row two doses of complement, and to all the tubes the proper amount of 5 per cent. blood suspension. The second incubation lasted one hour and the readings were found to be uniform throughout. The results are shown in table III, in which, however, in order to avoid unnecessary duplication, the results with two sera only are given.


\textsuperscript{17}Karsner, H. T., and Pearce, R. M., \textit{loc. cit.}
It is seen that in all the dilutions of dog serum used, the action of one dose of amboceptor and of complement was hindered, but in none of the dilutions was the antihemolytic property sufficient to hinder the action of two doses of complement and of amboceptor. Weaker dilutions were not considered necessary because it seems certain that between the action of a dilution of 1/64 on one dose of hemolysin and of whole serum on two doses, no fine gradation could exist.

In determining the complement value of dog serum, it was thought best to use a sheep hemolytic system. The anti-sheep amboceptor was titrated with normal dog complement and the approximate complement value determined. Then two series of tubes were arranged with one dose of amboceptor, the proper amount of 5 per cent. sheep blood suspension, and in the first row increasing doses of fresh serum from the six dogs. The second row was placed so that each tube contained corresponding ingredients to those in the first row except that no anti-sheep amboceptor was present. The latter series showed the natural amboceptor in the dog serum. It was found that the natural sheep hemolysin was the same in all except one. In spite of this the complement value was precisely the same in all six animals. The results with one animal are charted (table IV) to show the technique.

<table>
<thead>
<tr>
<th>Dog complement in c.c. (June 15, 1912)</th>
<th>0.01</th>
<th>0.02</th>
<th>0.04</th>
<th>0.07</th>
<th>0.1</th>
<th>1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (normal) without amboceptor ....</td>
<td>P.H.</td>
<td>H.</td>
<td>C.H.</td>
<td>C.H.</td>
<td>C.H.</td>
<td>C.H.</td>
</tr>
</tbody>
</table>
DISCUSSION.

These studies demonstrate conclusively that the increased resistance of the erythrocytes of the splenectomized dog depends upon the corpuscles rather than the serum. Pel's experiments demonstrated this fact so far as the resistance to hypotonic salt solutions is concerned, and the experiments reported in this communication not only confirm Pel's observations but add positive evidence of resistance to immune serum; and they demonstrate, moreover, that the serum of the splenectomized animal presents no change in complementary activity and no distinct increase in antihemolytic property. This demonstration is conclusive, for in the hemolysis in vivo as well as in vitro three components are necessary; the corpuscles, the amboceptor, and the complement. If anticomplementary substances were present in the blood, they would be effective in diminishing the complementary action of the animal's serum. If the complement itself were small in amount or diminished in activity, it would be seen in the results of the test-tube experiments. The amboceptor is the constant and controllable factor in the experiment and the only uncontrollable agent in this connection is an anti-amboceptor or antihemolysin, which, from the results of our experiments, is not found in the three splenectomized animals, or in the animal which had been given immune serum, in greater amounts than in normal dogs. The remaining factor is the red blood corpuscle itself; and that it is in this cell that the increased resistance lies is shown by these experiments, which demonstrate beyond question that the corpuscles of the splenectomized animals and of the animals which had been given immune serum exhibit distinctly increased resistance to the definitely controlled action of specific amboceptor and complement.

Why the absence of the spleen increases the resistance of the red cells is still an open question, but the information obtained by this study, excluding as it does changes in the serum, narrows the problem to the red cells themselves. Of great significance are the results of the study of the blood of dog 43. This blood was used as a control on the supposition that an administration of a hemolytic serum might have caused the production of an antihemolysin. But this was not demonstrable either in this dog or in dog 24, which also had received hemolytic serum.
The conclusion is unavoidable that the increased resistance of the corpuscles, after the use of such a serum, is associated with the anemia caused by the hemolytic serum. This increased resistance of red cells in anemia is not uncommon, and, as has been stated, has been brought forth by Bottazzi, in connection with the action of pyrodin, as an explanation of the increased resistance of the red cells to this drug after splenectomy. That the increased resistance may be due to splenectomy alone and be entirely independent of the action of hemolytic substance introduced from without, is shown in the experiments with the blood of dogs 51 and 46, and has also been clearly demonstrated in the first and third communications of this series. One cannot therefore escape from the conclusion that the increased resistance of the red cells is a concomitant of the regeneration of the blood which follows the anemia caused by the removal of the spleen. That anemia does follow splenectomy has been shown in many investigations, and recently has been demonstrated anew in this laboratory by Musser; 18 that the return of the blood picture to normal is accompanied by increased resistance of the red cells to hemolytic sera has been shown in the third communication of this series, in which also it has been demonstrated that anemia has an important bearing on the production of hemolytic jaundice.

The main problem therefore is the explanation of the anemia following splenectomy. The possibility that this anemia may be due to an autohemolysin normally neutralized by the spleen, but operative in the absence of the spleen, is excluded by the control experiments here presented. There remains therefore the question of how the spleen regulates or controls or is otherwise concerned in the general process of blood destruction and regeneration, and to obtain information on this point, the changes in the lymph nodes and bone marrow, after splenectomy, are now receiving especial consideration.

CONCLUSIONS.

1. The erythrocytes of splenectomized dogs show increased resistance to the action of hypotonic salt solutions and to specific

hemolytic immune serum. The degree of resistance appears to increase with the length of time that has elapsed after splenectomy.

2. This increased resistance of the erythrocytes is not due to an increased antihemolytic power of the animal's serum or to a diminished complementary value of the serum, but is a property depending upon the erythrocytes themselves.

3. Non-splenectomized animals receiving a single injection of specific hemolytic immune serum and developing a temporary anemia show likewise on recovery an increased resistance of the corpuscles without the presence of antihemolysin in demonstrable amount.

4. As anemia of varying grade is a characteristic result of splenectomy, it would appear that the increased resistance of the corpuscles is a concomitant of the regeneration of the red cells following such anemia and is thus analogous to the increased resistance of such cells not infrequently observed in various forms of experimental anemia.

5. There is no evidence to indicate that the anemia after splenectomy is due to the presence of hemolytic bodies, or that the increased resistance of the cells is due to antihemolytic bodies, accumulating in the serum as the result of the ablation of the spleen. It is evident therefore that the spleen in some way controls or regulates blood destruction (and regeneration ?), and in the hope of throwing light on the subject, an investigation of the bone marrow and lymph nodes of splenectomized dogs is now under way.