THE INFLUENCE OF ANEMIA ON PHAGOCYTIC FUNCTIONS IN RATS

By L. Joe Berry, Ph.D., and Evelyn C. Haller, A.B.

INTRODUCTION

An increase in the phagocytic activity of the neutrophiles in blood of anemic persons was observed in vitro by Berry, Davis, and Spies; this increase, which was observed in every type of anemia tested, varied between 120 per cent and 340 per cent of the value obtained with the blood of "normal" subjects. In several cases the authors were able to measure the phagocytic activity of the blood cells during relapse of the anemia and then to observe the changes that occurred with remission of the anemia following appropriate therapy. Curves 1 and 2 in their paper indicate that there is an approximate proportionality between the severity of the anemia and the percentage of increase in phagocytosis of the polymorphonuclear leukocytes. Berry, Leyendecker, and Spies in the course of a careful study of the method used in the earlier work, showed that the increase in the number of bacteria engulfed by the neutrophiles in anemic blood could not be caused by decreasing the number of red cells in normal blood to an anemic level nor by altering the oxygen tension in normal blood by means of various gas mixtures. It was further shown that the increase in phagocytosis was maintained by the leukocytes from anemic blood after they had been washed in a physiological solution of sodium chloride and returned to serum from either normal blood or anemic blood. Normal leukocytes, treated in a similar manner, exhibited essentially the same lower phagocytic activity in either the homologous serum or anemic serum. It was concluded, therefore, that the observed increase was due to a fundamental change of unknown character in the white blood cell rather than to some alteration in the anemic serum.

The present investigation was undertaken with albino rats in an effort to verify and extend the observations made with human blood. This reversal of the usual procedure seemed desirable in order to establish control over the experimental material. With this in mind, the animals were subjected to periodic blood loss, the phagocytic activity of the granulocytes was followed during this period and then observed after varying periods of recovery. The results obtained from the experimental work on the rats were found to confirm fully the original data described for the human.

METHODS

Sixty Sprague-Dawley male albino rats were used in these experiments and were fed a commercial ration of "rabbit pellets" ad libitum. These animals were 35 to 40 days old at the beginning of the experiment. Twenty of these rats were desig-
nated as the experimental group and were subjected to weekly bleeding for eleven weeks. At the end of this period the bleedings were increased to three a week for five weeks and the animals were then allowed to recover. Of the original 20 in the group, 10 survived throughout the experiment.

Bleeding was accomplished by cardiac puncture under light general ether anesthesia. The volume of blood removed was increased as the animals gained weight. At the beginning, approximately 1 cc. was withdrawn and toward the end from 2.5 to 4 cc. were removed. The latter figure was the amount desired, but the technical difficulties of the procedure made it impossible to bleed each animal to a uniform volume. The blood from the heart of half of the group was discharged quickly into a paraffined watch crystal, and 0.5 cc., measured in a tuberculin syringe, was added to 0.5 cc. of heparinized* saline for phagocytic measurements. Red cell counts, leukocyte counts, and hemoglobin determinations were also made with this blood. The blood from the remainder of the experimental animals was discarded. In other words, the data were obtained from one half of the group at one bleeding and from the other half of the group the next bleeding. During the period in which three bleedings were carried out a week, data were taken from the blood only once a week and the remainder of the blood discarded.

The 40 remaining rats were divided into 8 groups of 5 each to serve as controls. Each time data were obtained from the experimental animals, 5 controls were also bled and comparable determinations were made with the control blood. A new control group was used each week until all had been bled once. They were then used again in the same sequence. No control was bled oftener than every six weeks and the volume of blood removed was kept at approximately 1 cc. Deaths reduced the number in this group to 30.

Phagocytic measurements were made on the heparinized whole blood by the method of Cottingham and Mills. The accuracy and limitations of this procedure are discussed by Berry, Leyendecker, and Spies. The number of bacteria engulfed by at least forty granulocytes in each blood smear was counted and these values for all of the experimental animals were averaged each week to give the mean phagocytic activity for this group. The same procedure was followed to arrive at the mean phagocytic activity for the cells from the control group. Thus each figure shown is derived from at least 200 neutrophiles and in some cases from as many as 280 neutrophiles.

Hemoglobin was measured in grams colorimetrically by means of a Coleman Universal spectrophotometer, model 11, which had been calibrated by the method described by Harrow. Total erythrocyte and total leukocyte counts were made with Bureau of Standards pipettes and counting chamber cover glasses.

In order to obtain macrophages for study, an exudate from the peritoneal cavity was obtained by injecting 2 cc. of Nujol intraperitoneally into each of 5 experimental animals and 5 control animals. Forty-eight hours later as much exudate as possible was removed without sacrificing the rats. The exudate from each group was pooled and diluted in 10 cc. of a physiological solution of sodium chloride.

* The heparin used in this work was kindly supplied by Dr. Leo A. Pirk of Roche-Organon, Inc., Nutley, N. J.
It was then centrifuged, the supernatant liquid was discarded and the cells re-
suspended in 0.5 ml. of saline. The phagocytic activity of the macrophages was
determined by the same method as that used for the whole blood. The number
of bacteria in 100 macrophages was counted and averaged for both control and
experimental groups. These determinations were made at the end of the triweekly
bleedings and again after four weeks of recovery. Reticulocyte counts were made
at the same time, using the vital staining technic with 0.5 per cent brilliant cresyl
blue described by Levinson and MacFate.6

The effect of frequent anesthesia with ether on phagocytic activity was tested
by anesthetizing one group of 5 control rats, which were not bled, three times
a week for six weeks. At the conclusion of this period the phagocytic index, blood
counts, and hemoglobin determinations were made. An exudate was also obtained
from these animals and all values were compared with those for a control group
on the same day.

RESULTS

Changes in Phagocytosis with Weekly Bleeding. The first bleeding of the experimental
group was done on November 3, and one week later the first data were collected on
these animals along with those of a control group. These results are entered in
table 1A under the date of November 10. Columns 2 and 3 give, respectively, the
mean number of bacteria engulfed by the neutrophiles of the rats in the control
and experimental groups. These values are identical to the first decimal place and
the probable error for each figure is the same. Since the method gives an error of
10 to 15 per cent on duplicate samples, the average number of bacteria per leukocyte
is given only to the nearest tenth and the probable error is rounded off to the nearest
hundredth. Column 4 gives the per cent of "normal" phagocytosis, which is the
quotient, expressed as a percentage, of columns 3 and 2. The statistical significance
of the difference between the means for the experimental and for the control groups
has been calculated for each pair of values according to Croxton and Cowden,8
and is given in column 5. If the true difference between the means is assumed to be
zero, the figure in column 5 shows the probability of chance variations causing the
actual differences which occur between the means. This difference is obtained by
subtracting the number in column 3 from that in column 3. A probability of 5 in
100, or 5 per cent, is usually considered statistically significant, and a probability
of 1 per cent or less is highly significant. Thus the means for November 16 are
not sufficiently different to be significant, since the probability of chance causing
the difference is 32 per cent. It is not until the third week of bleeding (November
26) that the phagocytic activity of the experimental animals is reliably increased
above the control value. This period of time might be compared to that found neces-
sary by Cottingham and Mills7 for the reduction in activity of blood phagocytes
when rats were changed from an adequate to a deficient diet. The first change ap-
peared at the end of the second week and was completed by the end of the fourth.
Rats changed from a deficient to an adequate diet required the same time for the
recovery of their phagocytic activity. Cottingham and Mills interpreted their
results as suggesting that new granulocytes from the bone marrow were required
before a marked change in phagocytic activity could be observed. The similar timing evident in the data presented in the present paper might lend support to this hypothesis.

The total erythrocyte count, the total leukocyte count, and the hemoglobin content in grams are given for the control and experimental group for each date in columns 6, 7, 8, 9, 10, and 11. Each figure is the average for 5 animals. A com-

**Table 1. Phagocytic Activity of Rat Neutrophiles during the Development of and Recovery from Anemia**

<table>
<thead>
<tr>
<th>Date of experiment</th>
<th>Phagocytic activity as mean number of bacteria engulfed by</th>
<th>Per cent normal Phagocytosis Exp. Control</th>
<th>Statistical significance probability that difference in means is chance</th>
<th>Mean total erythrocytes in millions</th>
<th>Mean total leukocytes</th>
<th>Mean hemoglobin in grams</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov. 10</td>
<td>1.5 ± 0.09</td>
<td>1.5 ± 0.09</td>
<td>100</td>
<td>7.19 ± 0.21</td>
<td>8767</td>
<td>8825 ± 10.2</td>
</tr>
<tr>
<td>Nov. 16</td>
<td>4.6 ± 0.33</td>
<td>5.2 ± 0.27</td>
<td>115</td>
<td>8.17 ± 0.34</td>
<td>8120</td>
<td>6440 ± 15.3</td>
</tr>
<tr>
<td>Nov. 26</td>
<td>3.6 ± 0.37</td>
<td>7.5 ± 0.31</td>
<td>125</td>
<td>7.63 ± 0.34</td>
<td>5860 ± 13.95</td>
<td>13.87 ± 13.45</td>
</tr>
<tr>
<td>Dec. 7</td>
<td>6.1 ± 0.21</td>
<td>8.0 ± 0.24</td>
<td>131</td>
<td>6.82 ± 0.24</td>
<td>11,370 ± 12.34</td>
<td>10.79 ± 12.06</td>
</tr>
<tr>
<td>Dec. 14</td>
<td>6.5 ± 0.17</td>
<td>6.1 ± 0.24</td>
<td>131</td>
<td>6.82 ± 0.24</td>
<td>13,140 ± 14.45</td>
<td>10.91 ± 12.06</td>
</tr>
<tr>
<td>Dec. 21</td>
<td>3.2 ± 0.18</td>
<td>4.8 ± 0.19</td>
<td>131</td>
<td>7.69 ± 0.24</td>
<td>12,950 ± 11.63</td>
<td>10.81 ± 12.06</td>
</tr>
<tr>
<td>Dec. 28</td>
<td>3.6 ± 0.17</td>
<td>5.3 ± 0.21</td>
<td>131</td>
<td>6.89 ± 0.24</td>
<td>10,650 ± 10.83</td>
<td>10.82 ± 12.06</td>
</tr>
<tr>
<td>Jan. 4</td>
<td>4.4 ± 0.20</td>
<td>6.1 ± 0.26</td>
<td>131</td>
<td>7.54 ± 0.24</td>
<td>9,990 ± 11.307</td>
<td>10.93 ± 12.13</td>
</tr>
<tr>
<td>Jan. 11</td>
<td>7.2 ± 0.34</td>
<td>8.4 ± 0.33</td>
<td>131</td>
<td>7.40 ± 0.24</td>
<td>9,725 ± 10.317</td>
<td>10.83 ± 12.66</td>
</tr>
<tr>
<td>Jan. 18</td>
<td>8.0 ± 0.30</td>
<td>11.3 ± 0.34</td>
<td>131</td>
<td>7.40 ± 0.24</td>
<td>9,383 ± 11.383</td>
<td>10.44 ± 13.57</td>
</tr>
<tr>
<td>Jan. 15</td>
<td>6.5 ± 0.34</td>
<td>11.3 ± 0.35</td>
<td>174</td>
<td>8.75 ± 0.24</td>
<td>13,080 ± 10.141</td>
<td>10.21 ± 10.07</td>
</tr>
<tr>
<td>Feb. 1</td>
<td>3.4 ± 0.20</td>
<td>7.1 ± 0.27</td>
<td>212</td>
<td>7.89 ± 0.24</td>
<td>9,600 ± 8.600</td>
<td>11.06 ± 11.06</td>
</tr>
<tr>
<td>Feb. 8</td>
<td>3.0 ± 0.16</td>
<td>4.4 ± 0.25</td>
<td>147</td>
<td>7.34 ± 0.24</td>
<td>13,420 ± 11.860</td>
<td>14.4 ± 9.81</td>
</tr>
<tr>
<td>Feb. 15</td>
<td>4.8 ± 0.24</td>
<td>5.9 ± 0.24</td>
<td>147</td>
<td>8.01 ± 0.24</td>
<td>12,720 ± 10.013</td>
<td>14.89 ± 10.89</td>
</tr>
<tr>
<td>Feb. 22</td>
<td>5.2 ± 0.23</td>
<td>9.6 ± 0.28</td>
<td>185</td>
<td>7.09 ± 0.24</td>
<td>11,900 ± 9.870</td>
<td>13.97 ± 8.58</td>
</tr>
<tr>
<td>Mar. 6</td>
<td>5.9 ± 0.40</td>
<td>4.1 ± 0.18</td>
<td>71</td>
<td>8.18 ± 0.24</td>
<td>11,340 ± 9.280</td>
<td>14.86 ± 13.59</td>
</tr>
<tr>
<td>Mar. 12</td>
<td>6.3 ± 0.38</td>
<td>5.1 ± 0.20</td>
<td>71</td>
<td>8.18 ± 0.24</td>
<td>11,620 ± 9.990</td>
<td>14.89 ± 14.89</td>
</tr>
<tr>
<td>Apr. 10</td>
<td>7.8 ± 0.24</td>
<td>6.4 ± 0.21</td>
<td>82</td>
<td>8.43 ± 0.24</td>
<td>9,990 ± 9.990</td>
<td>14.83 ± 14.88</td>
</tr>
</tbody>
</table>

Comparison between groups shows that from one week until the next there had been a complete recovery of all blood cellular elements and also of normal hemoglobin values. There was, therefore, no evidence of anemia developing in these animals over the eleven week period of weekly bleedings. The phagocytic function of the granulocytes shows, however, a significant increase each week, beginning November 26 and continuing through January 18, except on December 14. There is no satisfactory explanation for the results obtained on this date, but there is the possibility that the reagents used for diluting the blood were improperly prepared.
The relatively small but significant difference between the control and experimental animals on January 11 is the exception to a 40 per cent to 50 per cent increase in phagocytosis during the remaining period. It should be emphasized that, in spite of every precaution to keep the procedure as rigidly uniform as possible, divergent measurements appeared at intervals. Even in the studies with human blood, Berry, Leyendecker, and Spies encountered similar difficulties, so that the present irregularities, though regrettable, are not unexpected, particularly since rat leukocytes clump more readily than human white blood cells and are present in smaller numbers in the blood smears.

**Phagocytic Changes with Triweekly Bleedings.** During the week beginning January 18, the animals were bled three times. This was continued for five weeks, or until February 22, and the results are given in table 1B. Under these conditions, as shown in column 4, the phagocytic activity in the experimental group increased. Here again, there is considerable variation from one week to the next, but higher percentages appear in most cases. Both the erythrocyte totals and the grams of hemoglobin undergo a definite and decided decrease in the experimental group when compared with the controls. The leukocyte totals, on the other hand, fall within normal limits for both groups even though the totals for the experimental animals are slightly lower in every case. These data suggest, therefore, that as the blood loss becomes more severe and as the animals develop an anemia the phagocytic function of the leukocytes increases. This corresponds to the observations made by Berry, Davis, and Spies with human subjects, previously referred to, that phagocytosis is roughly proportional to the severity of the anemia and is apparently independent of the leukocyte count.

**Phagocytic Changes During Recovery.** Following the five weeks of triweekly bleeding the animals were allowed to recover without blood loss for two weeks. At this time a volume of blood just sufficient for phagocytic measurements and blood counts was removed from one half of the experimental group. These data are entered in table 1C under the date of March 6. There is a significant drop in phagocytosis at this time, compared to the control animals, as shown by the 71 per cent value in column 4. At the same time, the erythrocyte total and hemoglobin content of the blood has returned to normal. This diminished function of the phagocytes is comparable to that found in human blood with the remission of anemia following appropriate therapy (see table 2 in paper by Berry, Davis, and Spies). In human
blood, phagocytic activity fell as low as 38 per cent of normal in 1 case and ranged up to 77 per cent in 5 of 6 cases reported. In the 1 case that failed to go below 100 per cent, it was clear from other data that the remission was not complete.

The remaining half of this group of rats were not bled again until four weeks after the last previous bleeding. The phagocytic activity measured on this date, March 22, was 84 per cent of the control, as shown in column 4. Essentially the same value, 82 per cent, was found on April 10 when the first half of the experimental group were bled again. The blood counts were normal each time. There is nothing in these data that would explain the decrease in phagocytic function over a six week period. Malnutrition is known to lead to a lowered activity of the white blood cells in rats (Cottingham and Mills and Berry, Davis, and Spies), and this was the explanation offered for the observation with the human anemias. The stock diet fed these animals is adequate for normal growth and development, but it may not be optimal for complete recovery within the period of the present investigation. On the other hand, the 16 per cent to 18 per cent decreases in phagocytosis shown on the last two dates are approaching the limits of reliability of

<table>
<thead>
<tr>
<th>Source of phagocytes</th>
<th>Phagocytic activity as mean number of bacteria engulfed by Experimental group</th>
<th>Per cent of normal phagocytosis</th>
<th>Statistical significance: probability that difference in means is chance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>7.8 ± 0.14</td>
<td>7.9 ± 0.17</td>
<td>101</td>
</tr>
<tr>
<td>Experimental group</td>
<td>0.7 ± 0.04</td>
<td>0.8 ± 0.05</td>
<td>114</td>
</tr>
</tbody>
</table>

the in vitro technic used in measuring this function. Additional data are required before an accurate evaluation of the decrease is possible.

In order to check the activity of the bone marrow during the bleeding and recovery of the experimental group, reticulocyte counts were made. On February 22, the anemic animals had an average of 40.7 per cent reticulocytes, and the controls had 5.9 per cent. After two weeks of recovery, the count for anemic animals had dropped to 8.7 per cent (March 6), and after four weeks it was 3.8 per cent (March 22). Figures for the controls on these dates were 4.9 per cent and 4.0 per cent. Creskoff, Fitz-Hugh, and Farris give the average adult reticulocyte count of rats as lying between 3 per cent and 4 per cent, but they state that normal values up to 10 per cent are found.

**Phagocytic Activity of Peritoneal Exudate.** Exudate was collected as described in the method at the end of the bleeding period on February 22, and again after four weeks of recovery on March 22. The results are shown in table 2. The probable error of the means and the statistical significance of the difference between the means are again presented. The 200 per cent of the control value shown in column 4 for February 22 may be compared with the 185 per cent found on the same date for whole blood in table 1B. Recovery was complete and the phagocytic activity was
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identical on March 22. The low absolute values for mean bacteria per macrophage given in columns 2 and 3 may be due to the fact that these measurements were made with washed cells resuspended in physiological solution of sodium chloride rather than in blood serum or tissue fluid.

Results with Etherized Controls. Table 3 gives the data obtained with the group of unbled animals anesthetized thrice weekly for six weeks and the same control group shown in table 1C for April 10. The mean number of bacteria per cell for whole blood shows a remarkably close agreement between the two groups. The results with the exudate obtained from the same animals are also essentially the same. It seems safe to conclude, therefore, that the results obtained in this study cannot be attributed to the changes in phagocytes induced by frequent ether anesthesia.

DISCUSSION

The parallelism between the changes in phagocytosis that accompany the development of and then the remission of anemia in the rat under experimental conditions and the observations by Berry, Davis, and Spies with cases of human anemia is most striking. In both studies the magnitude of increase in phagocytosis is approximately proportional to the severity of the anemia, and in both cases there is a decrease in phagocytosis to subnormal levels when the remission of the anemia is essentially complete. More recently (Berry and Haller) the leukocytes from the albino mouse have also been found to increase their phagocytic function as anemia develops from blood loss. With this evidence, combined with the demonstration by Berry, Leyendecker, and Spies of the apparent validity of the in vitro method under the conditions of these experiments, it seems reasonable to conclude that there is some connection between increased phagocytosis and anemia. The increase, furthermore, involves not only the microphages but also the macrophages, at least in the rat. This suggests that the cellular defense mechanism is increased either by the anemia or by the conditions that lead to the anemia. There is, at present, no evidence regarding these points. However, it is possible to test experimentally the protection that might be afforded an animal made anemic by blood loss. Controlled experiments by Berry and Haller have shown that mice are much more resistant to artificially induced Salmonella typhimurium infections under these conditions.

In speculating about the causal relationship between anemia and phagocytosis, several points should be clarified. Frank anemia, as such, is not necessary for a significant enhancement of phagocytic function, at least under the conditions of the experiments with the rats. There was, however, sufficient blood loss to give rise to a transitory anemia and to stimulate the bone marrow to activity. As blood loss was increased, it seems reasonable to assume that the bone marrow was forced to generate cellular elements at maximum rate. Under these conditions, phagocytic function increased still more. Thus it should be possible to link phagocytosis to bone marrow activity, as well as to the anemia. The studies with blood from cases of human anemia by Berry, Davis, and Spies make it unlikely, however,
that the rate of haematogenesis is involved. Numerous cases of macrocytic hyper-
chromic anemia were tested and since the marrow in these anemias is classically
characterized as hyperplastic with maturation arrest, and since leukopenia with
a marked right shift in the Arneth nuclear index occurs, there is no reason to
believe that rapid generation of blood leads to the increase in phagocytosis.
There is, on the other hand, consistent evidence that links the increase in phago-
cytosis to the anemic condition.

SUMMARY

1. Young male albino rats were subjected to weekly bleedings by cardiac
puncture for eleven weeks. Phagocytic activity of the granulocytes in this blood
was determined at each bleeding by a whole blood in vitro technic and compared
to that of the blood from a control group consisting of 5 animals. Total blood
cell counts and hemoglobin determinations were also made each week with both
groups. The control animals were not bled more frequently than once in six weeks.
2. Phagocytic function in the bled animals increased approximately 40 per cent
above the values for the controls during the period of weekly bleeding. There was
no evidence of anemia developing during this period.
3. The experimental animals were then bled three times weekly for five weeks.
Phagocytosis increased about 80 per cent above control values and the anemia be-
came severe. Macrophages obtained from a peritoneal exudate stimulated by in-
jections of mineral oil at the end of this five week period became approximately
twice as phagocytic as macrophages from control animals.
4. With the cessation of bleeding, there was a complete remission of the anemia
as measured after two weeks and the phagocytic activity of the blood had dropped
below normal. There was a partial return to normal within four weeks, but it
was still slightly subnormal at six weeks. The macrophages, on the other hand,
were normal when tested four weeks after recovery.
5. Control animals subjected to ether anesthesia thrice weekly for six weeks
showed normal phagocytic functions, as measured with blood and macrophages.
6. These results are discussed in the light of earlier experiments on the phago-
cytic activity of blood from anemic humans.

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