Anemia in Chickens with a Transplantable Lymphoid Tumor

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SUMMARY

Chickens inoculated with a transplantable lymphoid tumor developed a progressive anemia that was classified morphologically as normochromic and normocytic. The bone marrow showed normal cellularity with no evidence of tumor metastasis, and immature erythrocytes were not detected in the peripheral blood. (Increased serum transferrin was correlated with a decline in both packed cell volume and hemoglobin concentration, as well as with time postinoculation.) A positive relationship was observed between the degree of tumor growth, the reduction in both packed cell volume and hemoglobin, and the increase in serum transferrin. The direct Coombs' test on erythrocytes from tumor-bearing and control chickens was consistently negative. Certain similarities between the anemia in these chickens and that in many human cancer patients make the transplantable lymphoid tumor system a potentially useful model for comparative studies of the underlying mechanisms of anemia associated with lymphoreticular neoplasia.

INTRODUCTION

Anemia is a frequent complication of cancer, and the underlying mechanisms are often obscure (1, 2, 5, 12, 15, 16, 20, 24, 28). The literature is replete with articles concerning this problem in man. Bodey (2) recently reported that anemia was present in 60% of patients with disseminated cancer and in over 90% of patients with acute leukemia. In some cases, the cause of anemia may be readily apparent (26, 28) but, in many, the cause is obscure. Price and Greenfield (24) reviewed the subject and concluded that the events leading to anemia in many cases was unsolved.

A model system that could be readily manipulated for obtaining information concerning the underlying mechanism and the possible control of anemia in cancer is highly desirable. The recent discovery (8, 9) of increased serum transferrin in chickens with a rapidly proliferating TLT (22), which was maintained since 1937 by serial passage in chickens, led to the present studies of the effects of this tumor on the hematological parameters of inoculated chickens. Correlations between duration and extent of tumor growth, serum transferrin levels, and development of anemia are presented in this report.

MATERIALS AND METHODS

Experimental Animals. All of the animals used in this study were Athens-Canadian chickens (13), obtained at 1 day of age from the Poultry Disease Research Center, University of Georgia, Athens, Ga. The chickens were transferred to battery cages in a semiisolated building and were conditioned for 3 to 6 weeks before experimental studies were begun. During the conditioning period and throughout the experiment, all control and principal chickens were housed together and were given the same basal diet and management.

Source of Tumor Inoculum. The TLT was propagated by i.m. inoculation of 0.5 ml of minced tumor in the pectoral muscles of the chickens. This inoculum had been maintained by serial implant transmission. Tumorous material for the 1st experiment of this study was obtained aseptically from chickens (38 days of age) at 10 days post-TLT inoculation. Tumorous material for the 2nd experiment, obtained from chickens (31 days of age) at 9 days postinoculation, had been transplanted 4 consecutive times. The excised tumor was suspended in an equal volume of Rous-Turner 0.9% NaCl solution (11) and then was minced in a tissue homogenizer (VirTis Co., Gardiner, N. Y.) for 2 min at 5000 rpm. Chickens were inoculated with the tumor mince as soon as possible (usually within 30 min) after it was prepared.

Experimental Procedure. Two experiments were carried out. For the 1st experiment in which PCV, Hb content, and serum transferrin were determined, blood was collected by cardiac puncture from 85 chickens (24 days old) to serve as preinoculation controls (0 day). Chickens were divided into 1 major tumor group and 2 major control groups, as follows.

In Group 1, 50 chickens were given 0.5 ml of TLT i.m. in the right pectoral muscles and were then divided into 5 subgroups of 10 each. The chickens in these 5 subgroups were bled and killed by cervical disarticulation, 1 subgroup every 2 days beginning on the 5th day postinoculation.

The 15 chickens in Group 2 served as serial-bleeding controls and were bled with each respective tumor group at 5, 7, 9, 11, and 13 days postinoculation.

In Group 3, 20 chickens served as single-bleeding controls to prevent variability in hematological parameters attributable to the frequent bleeding of the serial controls in Group 2. The single-bleeding controls were divided into 4 subgroups of 5

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2 USPHS postdoctoral trainee in pathology.
3 The abbreviations used are: TLT, transplantable lymphoid tumor; PCV, packed cell volume; Hb, hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration.
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each and, at 7, 9, 11, and 13 days postinoculation, 1 of the 4 subgroups was bled, along with each respective tumor group and the serial controls. The single- and serial-bleeding controls were sacrificed on Day 13, along with the last tumor group.

Blood samples (1.5 to 2.0 ml) were collected from each chicken via cardiac puncture. One ml of blood was placed in a tube containing 10 μl of a 10% dipotassium EDTA solution (Cambridge Chemical Products, Inc., Detroit, Mich.), and the remaining blood was placed in a plain tube and was allowed to clot at room temperature. A postmortem examination was done on each group of tumorous chickens following their respective bleeding periods, and on all control groups at the termination of the experiment on Day 13. On the basis of gross observations at necropsy, tumorous activity in each chicken was graded as follows: Grade 0, no growth; Grade 3, growth and regression; Grade 5, local growth only; Grade 7, growth with localized metastasis; and Grade 10, growth with diffuse metastasis. The grade of tumor was subsequently verified by histopathological examination. Portions of tumor, liver, spleen, bursa of Fabricius, thymus, kidney, adrenal gland, gonad, proventriculus, brain, heart, and skin, plus the bone marrow from both femurs, were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Bone smears, prepared on coverslips, were obtained from tumor-bearing and control chickens at various intervals postinoculation and were stained with triple-strength Wright's, Wright-Giemsa, and new methylene blue stains (18). We examined smears of peripheral blood by counting a minimum of 40 oil immersion (X1000) fields (approximately 4000 erythrocytes) in order to determine the percentage of immature erythrocytes in the blood of control and tumorous chickens. In addition, a large volume of blood was removed from normal chickens, and the peripheral blood picture of a regenerative anemia case was compared with that of tumorous chickens at daily intervals after either blood withdrawal or TLT implantation. The morphological criteria for a regenerative response in the peripheral blood included the presence of anisocytosis, poikilocytosis, polychromasia, immaturity of nuclear chromatin, and increased numbers of reticulocytes, when stained with new methylene blue. Bone marrow smears, taken at multiple (2 to 4) sites per femur, were prepared immediately after death and were stained in the same manner. Myeloid:erythroid ratios were determined on selected bone marrow smears taken from tumorous and control chickens at each interval postinoculation. An estimate of bone marrow cellularity was obtained by examination of the hematoxylin- and eosin-stained sections.

A group of 25 chickens, 32 days of age, was used for the 2nd experiment. In addition to the above determinations, erythrocytic indices were determined for morphological characterization of the anemia. The direct Coombs' antiglobulin test (6) which used commercial rabbit anti-chicken globulin (3) that had been adsorbed with normal, washed chicken erythrocytes, was performed on erythrocytes from both tumorous and control chickens. This commercial antiserum reacted only with chicken globulins, as determined by immunoelectrophoresis. The titer of the Coombs' serum, determined by the Ouchterlony double-diffusion test in agar, was 1:4. Both positive (normal chicken erythrocytes sensitized with blood group isoantibody) and negative controls were utilized for an evaluation of the specificity of the antiglobulin reaction. Erythrocytes to be tested were washed 4 or 5 times in 0.9% NaCl solution until the supernatant was negative for protein on a commercial test strip (Labstix; Ames Co., Elkhart, Ind.). The packed cells were then made up to a 5% suspension with 0.9% NaCl solution. After 0.2 ml of Coombs' serum was added to 0.2 ml of cells, the mixtures were immediately centrifuged for 15 sec at 1000 RCF, and then were observed macroscopically and microscopically for agglutination. Next, the mixtures were incubated at 37° for 30 min and were observed again, after which they were refrigerated at 4° overnight before a final reading for agglutination was performed.

Chickens for the 2nd experiment were divided into 2 tumor groups, 1 serial-bleeding and 1 single-bleeding control group (Tables 1 to 3). Chickens with tumors were bled and necropsied, 5 at 7 days postinoculation and the remainder at 12 days postinoculation. Each of the 25 chickens was bled at Day 0 for preinoculation control samples. Procedures for blood collection, necropsy, and histopathology were as described for Experiment 1.

Hematological Studies. The RBC count was determined by electronic particle counting (Model B Coulter counter; Coulter Electronics, Inc., Hialeah, Fla.) according to the method of Stino and Washburn (29). Dilutions were made with an automatic Dade dual diluter (Dade Reagents, Inc., Miami, Fla.). Manual counting in a hemocytometer was also done with phloxin diluting fluid (21), and the precautions advised by Denington and Lucas (7) were followed. Manual and electronic particle erythrocyte counts were compared, since Coulter counting of nucleated chicken erythrocytes is not a routine procedure. Hb was determined by the cyanomethemoglobin procedure (29), and the PCV was measured by the procedure (29), and the PCV was measured by the

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Days after inoculation</th>
<th>PCV (%)</th>
<th>Hb (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10) &amp; TLT-inoculated (15)</td>
<td>0</td>
<td>32.12 ± 0.52</td>
<td>9.56 ± 0.19</td>
</tr>
<tr>
<td>TLT (5)</td>
<td>7</td>
<td>22.90 ± 1.01</td>
<td>6.72 ± 0.32</td>
</tr>
<tr>
<td>Serial control (5)</td>
<td>7</td>
<td>29.10 ± 0.81</td>
<td>9.12 ± 0.29</td>
</tr>
<tr>
<td>TLT (6)</td>
<td>12</td>
<td>16.20 ± 1.33</td>
<td>4.96 ± 0.36</td>
</tr>
<tr>
<td>Serial control (5)</td>
<td>12</td>
<td>26.75 ± 0.60</td>
<td>8.53 ± 0.30</td>
</tr>
<tr>
<td>Single control (5)</td>
<td>12</td>
<td>29.90 ± 1.03</td>
<td>8.70 ± 0.25</td>
</tr>
</tbody>
</table>

Table 1

Experiments 2: the packed cell volume and hemoglobin content in chickens inoculated in the pectoral muscles with 0.5 ml of TLT mince compared with noninoculated single- and serial-bleeding control chickens

The packed cell volume and hemoglobin content were determined by the microhematocrit and cyanometemoglobin methods, respectively. Data are expressed as mean ± S.E. Analysis of variance indicated significant (p < 0.01) decreases in the mean packed cell volume and hemoglobin values of the 7- and 12-day tumorous chickens when compared with the 0-day, single, and serial control means.
The erythrocyte numbers were determined by electronic (Coulter) counting and by manual counting in a hemocytometer. Data are expressed as mean ± S.E. Analysis of variance indicated a significant (p < 0.01) decrease in the mean erythrocyte numbers of the 7- and 12-day tumor chickens when compared with the 0-day, single, and serial control means. There was no significant difference (p > 0.05) between the means of the erythrocyte counts determined by electronic counting and those determined by manual counting.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Days after inoculation</th>
<th>Coulter counter</th>
<th>Hemocytometer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10)(^b) and TLT-inoculated (15)</td>
<td>0</td>
<td>1.96 ± 0.035</td>
<td>2.38 ± 0.041</td>
</tr>
<tr>
<td>TLT (5)</td>
<td>7</td>
<td>1.48 ± 0.109</td>
<td>1.58 ± 0.094</td>
</tr>
<tr>
<td>Serial control (5)</td>
<td>7</td>
<td>2.05 ± 0.063</td>
<td>2.01 ± 0.088</td>
</tr>
<tr>
<td>TLT (6)(^c)</td>
<td>12</td>
<td>1.23 ± 0.125</td>
<td>1.32 ± 0.100</td>
</tr>
<tr>
<td>Serial control (5)</td>
<td>12</td>
<td>2.18 ± 0.099</td>
<td>2.06 ± 0.087</td>
</tr>
<tr>
<td>Single control (5)</td>
<td>12</td>
<td>2.08 ± 0.038</td>
<td>2.26 ± 0.080</td>
</tr>
</tbody>
</table>

\(^a\) Erythrocyte number.
\(^b\) Numbers in parentheses, number of chickens in each group.
\(^c\) Four of 10 chickens inoculated with TLT died between Days 9 and 11.

Experiment 2: MCHC and MCV (calculated by the use of erythrocyte numbers determined by electronic and manual counting) in chickens inoculated in the pectoral muscles with 0.5 ml of TLT mince compared with noninoculated single- and serial-bleeding control chickens

Data are expressed as mean ± S.E. There was no significant difference (p > 0.05) in the MCHC means in any of the tumor and control groups studied. At 7 days postinoculation, there was no significant difference (p > 0.05) in the MCV means between the tumor and control groups, but at 12 days there was a significant difference (p < 0.01) with a shift toward microcytosis.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Days after inoculation</th>
<th>MCHC (%)</th>
<th>MCV (cu µm) determined by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10)(^b) and TLT-inoculated (15)</td>
<td>0</td>
<td>29.78 ± 0.34</td>
<td>164.51 ± 2.43</td>
</tr>
<tr>
<td>TLT (5)</td>
<td>7</td>
<td>29.41 ± 1.15</td>
<td>156.28 ± 5.04</td>
</tr>
<tr>
<td>Serial control (5)</td>
<td>7</td>
<td>31.33 ± 0.26</td>
<td>142.14 ± 3.30</td>
</tr>
<tr>
<td>TLT (6)(^b)</td>
<td>12</td>
<td>30.76 ± 0.68</td>
<td>132.59 ± 2.98</td>
</tr>
<tr>
<td>Serial control (5)</td>
<td>12</td>
<td>31.84 ± 0.45</td>
<td>122.86 ± 3.51</td>
</tr>
<tr>
<td>Single control (5)</td>
<td>12</td>
<td>29.12 ± 0.29</td>
<td>136.38 ± 4.81</td>
</tr>
</tbody>
</table>

\(^b\) Numbers in parentheses, number of chickens in each group.
\(^b\) Four of 10 chickens inoculated with TLT died between Days 9 and 11.

RESULTS

Experiment 1. There was a precipitous fall in PCV and Hb content over a 13-day period in chickens bearing TLT, compared with that in both serial- and single-bleeding controls (Charts 1 and 2). Although there was a slight decrease in these hematological parameters in the serial-bleeding control group, these chickens began to compensate at 9 to 11 days into the experiment. There was no significant compensation for the anemia in the tumor groups, even at 13 days postinoculation. Microscopic examination of peripheral blood from chickens with TLT revealed no increase in numbers of immature microhematocrit method (29). The MCV and MCHC were calculated.

Serum Studies. Serum transferrin was determined by a modification of an immunquantitation method for avian transferrin (8). Cellulose acetate membranes (Celotate; Millipore Corp., Bedford, Mass.) were used in conjunction with an all-plastic NIL-Saravis immunodiffusion apparatus (National Instrument Laboratories, Inc., Rockville, Md.).

Statistical Analysis. In both experiments, chickens were randomly assigned to the tumor group and the serial- and single-bleeding control groups. The data were statistically analyzed by an analysis of variance (27).
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erythrocytes at any interval postinoculation. The bone marrows of tumorous chickens were normocellular, compared with controls, and there was no evidence of invasion by tumor cells. The myeloid:erythroid ratios were also normal in the tumorous chickens.

In normal chickens from which a large volume of blood was removed, there were marked numbers of immature erythrocytes characterized by reticulocytosis (8 to 22%), macrocytosis, and polychromasia in the peripheral blood smears at 48 to 96 hr postremoval. This regenerative response ended 7 to 9 days after the blood withdrawal. There was also a slight increase in numbers of immature erythrocytes in the serial-bleeding controls at 7 to 13 days into the experiment. The single-bleeding controls (normal chickens) occasionally had a few (less than 2%) immature erythrocytes in their blood smears. Analysis of variance was used to compare the mean PCV and Hb content of 0-day controls and each respective single and serial control with the means of the 5-, 7-, 9-, 11-, and 13-day TLT groups. Statistical significance ($p < 0.01$) was found in all but the 5-day group. Comparison of

Chart 1. PCV at intervals postinoculation in chickens inoculated with 0.5 ml of TLT mince compared with noninoculated single- and serial-bleeding control chickens. PCV was determined by the microhematocrit method. *Numbers, the number of chickens from which blood was examined.

Chart 2. Hemoglobin content at intervals postinoculation in chickens inoculated with 0.5 ml of TLT mince compared with noninoculated single- and serial-bleeding control chickens. Hemoglobin content was determined by the cyanomethemoglobin procedure. *Numbers, the number of chickens from which blood was examined.
determinations at 0 day with each single-bleeding control group indicated no significant variance ($p > 0.05$).

There was an increase in serum transferrin levels in chickens bearing lymphoid tumors, compared with the single- and serial-bleeding controls (Chart 3). There were 2 chickens with normal serum transferrin levels. These were in the 11-day TLT group (Chart 3), and they had a Grade 5 tumor (local growth) and only a mild anemia. Analysis of variance was used to compare the mean serum transferrin of the 0-day preinoculation controls and each respective single-bleeding control group with the means of the 5-, 7-, 9-, 11-, and 13-day TLT groups. Statistical significance ($p < 0.01$) was found in each group. There was no difference ($p > 0.05$) between the mean of the 0-day control group and that of each single-bleeding control group.

There was a positive relationship between (a) the growth activity of TLT's and the reduction in PCV and Hb and (b) the increase in serum transferrin in chickens necropsied at 11 and 13 days postinoculation (Chart 4). As the growth activity of the tumors increased, the severity of anemia and the serum transferrin level increased.

**Experiment 2.** The hematological changes in chickens in Experiment 2 (Tables 1 to 3) were comparable to those of Experiment 1. An analysis of variance indicated no significant difference ($p > 0.05$) between the 0-day and single-bleeding control means but it indicated highly significant ($p < 0.01$) decreases in the mean PCV, Hb, and RBC counts in the 7- and 12-day TLT chickens, compared with the 0-day and single- and serial-control means. However, there was no significant difference ($p > 0.05$) in the MCHC means in any of the tumor or control groups studied, thus indicating a morphological classification of normochromic anemia. At 7 days post-TLT, there was no significant difference ($p > 0.05$) in the MCV means in any of the tumor or control groups, but at 12 days there was a significant difference ($p < 0.01$) between the tumor and preinoculation (0 day) control groups. However, there was also a shift toward microcytosis in the single and serial noninoculated control groups. There was no significant difference ($p > 0.05$) between the means of the RBC values determined by electronic counting and those mean values determined by manual counting.

The direct Coombs' test on erythrocytes obtained from each tumor-bearing and control chicken at 0, 7, and 12 days postinoculation was negative.

The gross and microscopic characteristics of the TLT were...
similar to those reported by Olson (22). The spleens of chickens bearing TLT were usually enlarged and very friable, on gross examination. On histological examination, sheets of large lymphoid cells with large vesicular nuclei and a scant basophilic cytoplasm were seen infiltrating between skeletal muscle fibers at the site of implantation. Only slight to moderate amounts of hemorrhage were seen, usually at the periphery of the tumor. A variable amount of necrosis was seen at the tumor implantation site. The necrotic zones were usually confined to the margin between the tumor and the viable tissue and to the surface of the pectoral muscles. Many localized metastatic foci, not seen at necropsy, were found upon histological examination of the viscera. A moderate increase in a brown pigment presumed to be hemosiderin was seen in the spleen but not in the tumor, liver, or bone marrow from chickens with TLT, compared with controls. There was no evidence of extramedullary hematopoiesis.

Chickens bearing lymphoid tumors continued to eat and drink throughout the experiments. At necropsy, the crops were filled with food and the chickens appeared well nourished.

DISCUSSION

The data presented show that chickens with a TLT develop a severe acute anemia over a period of 12 to 14 days that can be classified morphologically as normochromic and normocytic. The increase in erythropoietic activity displayed by the bone marrow and peripheral blood of normal chickens after acute hemorrhage or massive blood withdrawal (30) was not seen in chickens with TLT. Estimates of cellularity from hematoxylin- and eosin-stained sections of bone marrow from tumorous chickens indicated a normocellular marrow with no evidence of erythroid hypoplasia or invasion by neoplastic cells. The myeloid:erythroid ratios were normal in tumorous chickens at each interval postinoculation. Lack of a regenerative response in the peripheral blood of tumorous chickens may be due to the fact that they die too quickly (12 to 15 days) for adequate appraisal of a marrow response, i.e., physiological adjustment to the anemia may not have taken place. However, we do not favor this idea, since the life-span of erythrocytes in chickens is reported to be about 28 days (18), and erythropoiesis can be mobilized much more rapidly in chickens than in mammals (30). Wirth and Kubasta (30) found that regeneration of anemia in chickens after acute blood withdrawal was vigorous, ending in about 1 week, compared to about 3 weeks for the same result in mammals. Lucas and Jamroz (18) reported that reticulocytes were extremely rare in normal adult chickens. However, upon massive bleeding, marked numbers of immature erythrocytes were found in the peripheral blood. Manipulation of the TLT system to allow longer periods of tumor growth would be helpful in further evaluating the marrow response in chickens with TLT.

Reports concerning the morphological classification of anemia in human cancers are variable (10, 14, 19). However, except in cases of blood loss, the majority of human cancers have a normochromic normocytic type and, in many cases, there is complete absence of a bone marrow response in the peripheral blood (5, 20, 26). Miller et al. (20) found no correlation between the presence of anemia and bone marrow cellularity and the extent of bone marrow metastases (if any) in many human cancer cases. Certain similarities exist between these findings in human cancers and those in chickens with TLT.

Hemorrhage into the tumor mass would be a simple mechanism to explain acute anemia in chickens with TLT, but this explanation appears unlikely, since hemorrhage was not present in most tumors and when observed was minor on gross and microscopic examination. In no case was massive hemorrhage present. The presence of autoimmune hemolysis [seen in some patients with cancer (17, 25)] in chickens with TLT is improbable, since the direct Coombs’ test with rabbit anti-chicken globulin was negative in both tumor and control groups in this study. However, this finding does not eliminate extracorpuscular hemolysis due to other hemolytic factors, nor does it eliminate the possibility of cell-bound immunoglobulin not detectable by the Coombs’ test. Possibly, a hemolytic and/or marrow depression factor is released, either from necrotic material in the tumor mass or from the tumor cells themselves. The morphology of the tumor may favor a stasis of erythrocytes within vascular areas that increases their exposure to lytic substances, but this is only speculative. In some severely anemic TLT chickens, only slight to moderate amounts of necrotic material were found. The normal bone marrow cellularity and myeloid:erythroid ratios of the tumorous chickens would suggest a defect in cell release instead of in the erythroid elements produced by the marrow. Ferro- and erythrokine assessments should prove useful in determining these possible mechanisms.

The significance of the rise in serum transferrin in chickens with TLT is unknown. Such an increase could result from transferrin production by TLT cells. A similar transferrin increase was reported in mice bearing transplantable tumors (4). This is in contrast to the usual finding in most human patients with neoplasia, in which transferrin is normal or is decreased in amount.

The most striking similarities between the anemia in chickens with TLT and that in many human cancer patients were a normochromic and normocytic anemia characterized by normal cellularity of the bone marrow and no evidence of marrow metastasis, yet failure of erythropoiesis. These similarities, coupled with the ready availability and ease of manipulation of this model, make the TLT system potentially useful for comparative studies on the anemia associated with lymphoreticular neoplasia.

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REFERENCES


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