Radioiron Incorporation by a Transplantable Lymphoid Tumor

Francis W. Chandler, Jr., and Oscar J. Fletcher, Jr.

Department of Veterinary Pathology and the Institute of Comparative Medicine, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30601

SUMMARY

The in vivo uptake of iron by tissues in chickens inoculated with a transplantable lymphoid tumor was studied, and 59Fe-labeled transferrin was used as a tracer. The transplantable lymphoid tumor incorporated 59Fe at a rate that was approximately 3 times that of the spleen and three-fourths that of the liver when 59Fe uptake was related to organ weight at 24 hr postinjection. Autoradiography at the light microscopic level showed that 59Fe was present in the lymphoid tumor cells and was not present in the interstitial, vascular, or necrotic areas of the tumor at either 24 or 72 hr after the injection of radioiron. The bone marrow, spleen, and bursa of Fabricius showed no increase in label above background. Tissue sections stained with Perls' reagent revealed little or no hemosiderin in the tumor or bone marrow but revealed moderate to marked increases in the amount of hemosiderin in the spleen and liver of most of the chickens that were inoculated with tumor. Lymphoid tumor-bearing chickens developed a progressive hypoferremia, and there was a precipitous drop in the packed cell volume. An increase occurred in both serum iron-binding capacity and transferrin levels over a 12-day period following tumor implantation. The uptake of iron by transplantable lymphoid tumor cells and an apparent blockage of iron reutilization were responsible for the rapid decline in serum iron.

INTRODUCTION

Although the occurrence of hypoferremia in certain forms of cancer in man has been reported by a number of investigators (1, 3, 21, 27, 31), the underlying mechanisms remain obscure. SI levels in cancer patients are usually low despite increased tissue iron stores (2, 4, 21, 27), and the anemia seen in these patients is not usually corrected by p.o. or parenteral iron therapy (24, 26, 29).

Robbins and Pederson (32) demonstrated a crucial role for iron in initiating and maintaining DNA synthesis during mitosis of HeLa cells. Walters et al. (36) found that immature leukemic cells from patients with acute leukemia possessed iron-incorporating activity in vitro, whereas leukocytes from healthy adult volunteers lacked this capacity. These findings indicate that tumor cells have an essential requirement for iron and suggest that such cells may serve as iron traps, thereby contributing to the hypoferremia of cancer. We investigated this hypothesis by examining the ability of cells of a chicken TLT (30) to incorporate radioiron. Chickens inoculated with this transplantable tumor (Olson) develop a progressive anemia and may serve as useful models for the study of cancer-associated anemia (8). The tissue quantitation and autoradiographic demonstration of the in vivo incorporation of radioiron by these lymphoid tumor cells from 59Fe-labeled transferrin are presented in this report. Changes in SI, transferrin, and iron-binding capacity are also presented.

MATERIALS AND METHODS

Experimental Animals

Athens-Canadian chickens (20), obtained at 1 day of age from the Poultry Disease Research Center, University of Georgia, Athens, Ga. were conditioned in battery cages in a semisolated room for 5 weeks before experimental studies were begun. Control and principal chickens were housed together and received the same basal diet and management throughout the conditioning and experimental periods.

Source of Tumor Inoculum

The TLT was propagated by inoculation of 0.5 ml of minced tumor i.m. in the pectoral muscles. This TLT has been maintained by serial implant transmission since 1937 (30). The excised tumor was suspended in an equal volume of Rous-Turner 0.9% NaCl solution (17) and minced in a tissue homogenizer (VirTis Company, Gardiner, N. Y.) for 2 min at 5000 rpm. Chickens were inoculated as soon as possible (usually within 30 min) after preparation of the inoculum.

Trial 1

Experimental Procedure

When 5 weeks old, 55 chickens were bled (1.5 to 2.0 ml) by cardiac puncture to supply serum and whole, uncotted (EDTA) blood for the preinoculation (Day 0) controls. The chickens were then divided into 1 tumor and 2 control groups as follows:

Group 1. Thirty-five chickens received 0.5 ml of TLT in the right pectoral muscles. Nineteen chickens were bled and killed by cervical disarticulation at Day 7, and the remainder were killed at Day 12 following TLT implantation.
Group 2. Fifteen non-TLT-inoculated chickens served as serial-bleeding controls and were bled at the same time intervals as was each respective tumor group.

Group 3. Five non-TLT-inoculated chickens served as single-bleeding controls for an evaluation of the variability in SI, TIBC, and transferrin values due to repeated bleeding of the serial controls. These controls were bled at the termination of the experiment.

A postmortem examination was done on tumor-bearing chickens killed at Days 7 and 12 postinoculation and on all control chickens at the termination of the experiment. Portions of tumor (inoculation site), bone marrow, liver, and spleen were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 µm, and stained with Perls’ reagent (25) and hematoxylin and eosin.

SI and TIBC

All blood samples (taken in midafternoon) were allowed to clot at room temperature; they were held at 4⁰ overnight, and then the serum was removed. SI and TIBC were determined with a commercial kit (Ferro-chek; Hyland Laboratories, Costa Mesa, Calif.) (33). Since 2 ml of serum were required for both of these tests, the Day 0 preinoculation and the serial control sera were pooled into 3 to 14 samples each, and a total of 7 pools were tested for the Day 0 control group and 3 pools each were tested for the 7- and 12-day serial-bleeding control groups. A commercial quality control product (Moni-trol I, Lot LTD-106C,D; American Hospital Supply Corporation, Miami, Fla.) was used to check the accuracy of this procedure. The coefficient of variation for repeated determinations (9 total) on the commercial control product was ±9% for SI and ±5% for TIBC.

Serum Transferrin

Serum transferrin was determined on 25 (11 principals and 14 controls) of the 55 chickens studied. We used a modification of an immunoquantitation procedure for avian transferrin (14), with cellulose acetate diffusion strips (Celotate; Millipore Filter Corporation, Bedford, Mass.) contained in a plastic NIL-Saravis immunodiffusion apparatus (National Instrument Laboratories, Inc., Rockville, Md.). Antisera was produced by the injection of albino rabbits with conalbumin (Nutritional Biochemicals Corporation, Cleveland, Ohio). Conalbumin, the iron-binding protein of egg white, is immunologically identical to avian serum transferrin (37). The anticonalbumin produced a single precipitani line, when allowed to react against chicken serum previously subjected to electrophoresis. Standards were run with each immunodiffusion strip, and the reproducibility of results was checked by repeated determinations of the standards and test sera.

The TIBC:transferrin ratios were calculated from the mean TIBC and transferrin values (Table 1) of all control and TLT groups.

PCV

One-half ml of blood from each animal was placed in a tube containing 5 µl of a 10% dipotassium EDTA solution (Cambridge Chemical Products, Inc., Detroit, Mich.). The PCV was determined on these EDTA blood samples by the microhematocrit method (35).

Statistical Procedure

Chickens were randomly assigned to the control and tumor groups. The statistical significance of differences between 2 groups was determined by an analysis of variance, and the probability of these was determined with a standard table of F values (34).

Trial 2

In view of the results in Trial 1, we examined the possibility that iron uptake by rapidly dividing TLT cells might be a mechanism at least partially responsible for the observed SI changes in tumor-bearing chickens.

Tracer. Ferrous citrate-59Fe (Ferrutope; E. R. Squibb & Sons, New Brunswick, N. J.) with a specific activity of 2.47 µCi/µg was used. Forty µCi were added to 8 ml of pooled fresh serum taken from normal chickens, and the mixture was incubated at 37⁰ for 30 min in order to allow binding to transferrin. The mixture was gently mixed several times during the incubation period. In order to ensure that only 59Fe bound to transferrin was used as a tracer, the mixture was then passed through an anion-exchange resin (IRA 400 CI⁻; Arthur H. Thomas Company, Philadelphia, Pa.) column (7) to remove any unbound iron that might cause a nonrepresentative distribution pattern in the body, i.e., a rapid primary hepatic uptake of unbound iron (13). The 59Fe-labeled transferrin was collected in the eluate and then passed through a 0.45-µm Millipore filter. We calculated the percentage recovery (59Fe binding) by counting the radioactivity in duplicate aliquots of the mixture obtained before and after passage through the anion-exchange resin. Each aliquot was brought to a uniform volume, and at least 10,000 counts/sample were counted in a Model SC-57 well-type sodium iodide scintillation counter (Tracerlab, Inc., Waltham, Mass.) attached to a Model 530S γ spectrometer (Baird-Atomic, Inc., Cambridge, Mass.). Pre- and postcolumn aliquots were counted at the same time in order to avoid correcting for physical decay.

Autoradiography. Ten chickens weighing between 340 and 465 g and bearing lymphoid tumors that had been implanted in the right pectoral muscles 6 days before, together with 4 nontumorous control chickens, received intracardial injections of 59Fe-labeled transferrin. Each animal received approximately 1 µCi of 59Fe per/200 g of body weight. The volume injected varied from 0.43 to 0.58 ml. Four additional tumor-bearing animals that did not receive radioiron served to supply tissues for use as background controls for autoradiography. Seven radioiron-injected chickens were killed by exsanguination at 24 hr postinjection, and the remaining 7 chickens plus the controls that did not receive radioiron were killed at 72 hr postinjection. Immediately thereafter, slices of tumor 1 to 2 mm thick were taken from representative areas and fixed in 10% neutral buffered formalin for 24 to 48 hr. Portions of the liver, spleen, femoral bone marrows, bursa of Fabricius, and noninjected pectoral muscle were also taken and fixed in the same manner.
Autoradiograms of tumor and other tissues were prepared by a modification of the technique of Murphy et al. (28). After fixation for 24 to 48 hr in formalin, tissues were dehydrated in increasing concentrations of aqueous alcohol, cleared in xylene, embedded in paraffin, sectioned at 5 μm, mounted on microscope slides, deparaffinized by heating in an oven at 60°C, and stained with hematoxylin. After the last absolute alcohol bath, the slides were dipped into 0.3% celluloidin dissolved in a 1:1 absolute alcohol:ether solution and air-dried overnight at room temperature. In a darkroom, the slides were dipped into Kodak NTB-3 emulsion (Eastman Kodak, Rochester, N. Y.) that was liquefied at 45°C and diluted 1:1 (v/v) with distilled water. The slides were then allowed to air dry for 1 hr and were stored at 8°C for 3 weeks in light-tight boxes containing desiccant granules. Following exposure, the autoradiograms were developed (Kodak D-19) for 6 min, fixed (Kodak Rapid Fixer) for 6 min, and washed in distilled water for 20 min. All photographic solutions were freshly prepared, filtered, and maintained at 8-10°C during processing in order to avoid artifacts, as described by Murphy et al. (28). The autoradiograms were then removed from distilled water and were immediately dehydrated in absolute alcohol and xylene. Coverslips were applied with the use of Permount (Fisher Scientific Company, Fair Lawn, N. J.).

Background grain counts were made over adjacent areas of the slides free of labeled tissue and on tissues from the tumor-bearing animals that did not receive radioiron. The grain count of the background averaged 11 grains/oil immersion (×800) field. Any cell with a greater number of grains than the background count over any part of the cell was considered labeled. A clean slide coated with emulsion was exposed to light as a positive exposure control for emulsion and processing reagents.

Tissue Quantitation of 59Fe. Twelve chickens that had received TLT 5 days earlier and 7 nontumorous control chickens received intracardial injections of 1 μCi 59Fe-labeled transferrin per 100 g of body weight. At 24 and 72 hr postinjection, designated groups (Table 2) of chickens were exsanguinated via cardiac puncture and necropsied. The tumor, spleen, liver, bursa of Fabricius, a portion of the intestine, muscle, and bone marrow from both femurs were removed from each chicken. All tissues, except bone marrow, were thoroughly washed in cold Rous-Turner 0.9% NaCl solution (17), blotted dry, weighed, and placed in separate glass tubes for radioassay. Random tissues were taken after washing and were checked for residual erythrocyte content by histological examination. The 59Fe content was determined in a well-type scintillation counter as described above. Background counts were subtracted, and the radioactivity per g of tissue and/or per organ was expressed as a percentage of the injected dose as determined from a preinjection 59Fe standard. All samples were counted at the end of the experiment in order to avoid correcting for physical decay, and sufficient counts were recorded to give a counting error of <3%.

RESULTS

Trial 1

The SI and PCV values at Days 7 and 12 postinoculation in chickens inoculated with TLT are presented (Chart 1). There was a progressive decrease in SI and PCV in tumor-bearing chickens.

The TIBC and serum transferrin values at Days 0, 7, and 12 following inoculation of chickens with TLT are presented (Table 1). Fig. 1 illustrates the radial diffusion patterns obtained when anticonalbumin was reacted against sera from 3 preinoculation control chickens and 3 chickens with TLT at Day 12 postinoculation. There was an increase in TIBC and serum transferrin which paralleled tumor growth. Statistical differences (p < 0.01) were found when the mean SI and PCV values of the 0-day group and of each respective serial- and single-bleeding control group were compared with those of the 7- and 12-day TLT groups. Comparison of determinations at Day 0 with each single- and serial-bleeding control group indicated no significant variance (p > 0.05). There was no significant difference (p > 0.05) between the mean TIBC of the 0-day, serial-, single-bleeding control, and 7-day TLT groups, but a statistical significance (p < 0.01) was found when the 0-day and serial-, and single-bleeding control groups were compared with the 12-day TLT group.

There was a positive correlation between the TIBC and serum transferrin levels in chickens bearing TLT, and each increased with time, postinoculation. However, the
**Table 1**
The TIBC and transferrin values at Days 7 and 12 after inoculation of TLT in the pectoral muscle, compared with noninoculated single- and serial-bleeding control chickens

Data are expressed as the mean ± S.E. (see text for statistical evaluation). The mean preinoculation (Day 0) TIBC value of the combined groups was 243.4 ± 14.3, and the mean preinoculation transferrin value was 151.4 ± 4.8.

<table>
<thead>
<tr>
<th>Group</th>
<th>TIBC (μg/100 ml) at Day 7</th>
<th>TIBC (μg/100 ml) at Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-bleeding control</td>
<td>200.0 ± 25.8 (3P)</td>
<td>222.5 ± 44.4 (3P)</td>
</tr>
<tr>
<td>Serial-bleeding control</td>
<td>284.8 ± 16.2 (19)</td>
<td>351.6 ± 18.1 (14)</td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Transferrin values (mg/100 ml) at Day 7</th>
<th>Transferrin values (mg/100 ml) at Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-bleeding control</td>
<td>140.4 ± 14.7 (5)</td>
<td>125.0 ± 6.1 (5)</td>
</tr>
<tr>
<td>Serial-bleeding control</td>
<td>242.5 ± 29.8 (3P)</td>
<td>322.5 ± 37.8 (6)</td>
</tr>
</tbody>
</table>

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**Lymphoid Tumor Uptake of Radioiron**

Distribution of $^{59}$Fe in Other Tissues. Grains were no greater than background over the spleen, bursa of Fabricius, and noninjected (normal) pectoral muscles of both tumor-bearing and control chickens. Moderate numbers of grains were seen over the erythroid cells in the bone marrows of nontumorous controls, whereas only occasional grains were seen over the erythroid cells of tumor-bearing chickens. There were moderate to marked numbers of grains over the cytoplasm of hepatic cells of all of the animals at each interval post-radioiron injection, whereas grains were no greater than background over the hepatic sinusoids, central veins, and portal triads.

Autoradiographic Controls. The number of grains over tissues from animals that did not receive radioiron was no greater than the background grain counts in autoradiograms from chickens that received radioiron.

Tissue Quantitation of $^{59}$Fe. The percentage of injected $^{59}$Fe/g, wet weight, of tissue from tumor-bearing and nontumorous chickens at 24 and 72 hr postinjection is shown in Table 2. The 24-hr bone marrow $^{59}$Fe incorporation in tumor-bearing chickens was significantly less ($p < 0.01$) than that in normal controls. At 72 hr postinjection, there was an increase in the uptake of $^{59}$Fe/g of liver and spleen, while the uptake of $^{59}$Fe/g of TLT remained relatively constant. One g of TLT accumulated approximately 15 times as much $^{59}$Fe as did the bursa of Fabricius, a lymphoid organ with cells morphologically similar to those of the tumor. When $^{59}$Fe uptake was related to organ weight at 24 hr postinjection, the entire TLT incorporated $^{59}$Fe at a rate that was approximately 3 times that of the spleen and three-fourths that of the liver.

**DISCUSSION**

TLT cells of chickens incorporated radioiron from $^{59}$Fe-labeled transferrin in vivo. Results of the tissue quantitation studies indicate that the TLT has a major role in plasma $^{59}$Fe uptake. Chickens bearing these tumors developed a progressive hypoferremia and anemia that paralleled tumor growth. These observations lend support to the hypothesis that neoplastic cells may act as iron traps and may contribute to hypoferremia and anemia in cancer. Numerous reports (22, 24, 26, 29) have described the association of lymphoreticular tumors and refractory anemia accompanied by hypoferremia in humans, especially children. Iron incorporation in vitro (but not to our knowledge in vivo) by human neoplastic cells has not been investigated.
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Table 2
The distribution of radioactivity in tumor-bearing and normal control chickens at intervals post-\(^{59}\)Fe injection

\(^{59}\)Fe was injected 5 days after tumor implantation, and data are expressed as the percentage of the injected dose per g. wet weight, of tissue.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Normal controls (3) (^a)</th>
<th>Tumor-bearing (5)</th>
<th>Normal controls (4)</th>
<th>Tumor-bearing (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>14.71 ± 1.12</td>
<td>0.31 ± 0.02 (^b)</td>
<td>3.19 ± 0.26</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>3.64 ± 0.32</td>
<td>0.32 ± 0.04</td>
<td>0.55 ± 0.05</td>
<td>0.62 ± 0.07</td>
</tr>
<tr>
<td>Liver</td>
<td>0.36 ± 0.05</td>
<td>0.43 ± 0.04</td>
<td>0.59 ± 0.03</td>
<td>0.88 ± 0.11</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.26 ± 0.03</td>
<td>0.02 ± 0.002</td>
<td>0.04 ± 0.007</td>
<td>0.04 ± 0.003</td>
</tr>
<tr>
<td>Bursa</td>
<td>0.03 ± 0.004</td>
<td>0.02 ± 0.003</td>
<td>0.02 ± 0.001</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>Pectoral muscle</td>
<td>0.02 ± 0.002</td>
<td>0.02 ± 0.003</td>
<td>0.02 ± 0.001</td>
<td>0.01 ± 0.001</td>
</tr>
</tbody>
</table>

\(^a\) Numbers in parentheses, number of animals.
\(^b\) Mean ± S.E.

been reported (32, 36).

There is some evidence that iron may be important for rapid tumor growth. Robbins and Pederson (32) demonstrated high concentrations of iron in interphase nucleoli and mitotic chromosomes of HeLa S\(_2\) cells grown in monolayers and suspension cultures. When iron was removed from the culture media with a chelating agent, inhibition of DNA synthesis and mitosis was observed. Their data suggest that iron is important in the initiation and maintenance of DNA synthesis. A similar mechanism may be responsible for an increased iron demand by the rapidly dividing TLT cells, and this possibility warrants further consideration. Additional evidence that iron may be needed for rapid division of neoplastic cells was provided by the \textit{in vitro} studies of Walters et al. (36). They found that polymorphonuclear peritoneal exudates from rabbits as well as leukocytes from healthy human adults and from patients with chronic leukemia could not incorporate radioiron into heme. However, immature leukemic cells from patients with acute leukemia possessed iron-incorporating activity. The authors did not determine whether the heme-synthesizing capability of immature leukemic cells was a function of their immaturity or of their neoplastic transformation. Burger and Lajtha (5) reported an increased uptake of radioiron in the liver and spleen from mice in which AKR leukemia was progressing. It was suggested, but not demonstrated, that iron incorporation into leukemia cells might account for some of the increase of radioactivity in these organs.

The uptake of \(^{59}\)Fe by chicken TLT cells and a reduced \(^{59}\)Fe uptake by erythroid elements of the bone marrow suggests that the neoplastic cells have a greater affinity for iron. Erythroid cells have not been recognized as a component of the TLT by us or by other investigators (6, 30). Although the TLT cells appear morphologically as lymphoblasts, they might have pluripotential qualities with the ability to synthesize heme. The decreased \(^{59}\)Fe incorporation by the bone marrow in tumor-bearing chickens could also be caused by a selective erythroid depression of iron uptake due to toxic factors secreted by the tumor cells and/or necrotic foci within the tumor. The results of this study do not permit a choice between these possible mechanisms.

In chickens bearing TLT, stains for hemosiderin revealed little or no storage iron in the tumor or bone marrow, but showed increased iron stores in the spleen and liver of most animals. Absence of stainable iron within the TLT cells or any other area of the tumor would indicate that iron is not present in the form of hemosiderin. Moderate to marked amounts of stainable iron in the reticuloendothelial cells of the spleen and liver from TLT chickens suggest a blockage of reutilization of the iron from senescent erythrocytes phagocytized by these cells, which would contribute further to the hypoferremic state. Defective reutilization of iron in cancer and inflammation was demonstrated by Haurani et al. (18, 19), and this mechanism was also proposed to explain the hypoferremia that developed following turpentine-induced inflammation in dogs (15) and after the injection of endotoxin in rats (23). The small foci of hemorrhage associated with the TLT in this study may also contribute to the hypoferremia, since nonreticuloendothelial iron is poorly reutilized (13).

The hypoferremia, anemia, lack of stainable marrow iron stores, and increased iron stores in the spleen and liver of tumor-bearing chickens somewhat parallel the findings of lymphoreticular neoplasia in man (11, 12, 16). Elevation of the TIBC as observed in chickens bearing TLT is extremely uncommon in human cancers, in which case the TIBC is usually depressed or normal (16, 31). Increased serum transferrin has been reported in mice bearing transplantable tumors (9, 10). Possible explanations for increased transferrin in chickens with TLT have been discussed previously (14).

The reason for the discrepancy in the TIBC:transferrin ratios between normal controls and tumor-bearing chickens is not known. One possible explanation is the production of a different molecular species of transferrin by the rapidly proliferating cells which is unable to bind iron. Electrophoretic and fluorescent antibody techniques may be helpful in answering this question. It has been reported that oviductal synthesis of conalbumin in egg-laying hens can contribute to elevations in the serum TIBC (37). The chickens used in this study were sexually immature (5 weeks old). In addition, similar TIBC and transferrin values were obtained in both male and female chickens, which would indicate that production of conalbumin by the oviduct was not a factor in these observations.

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REFERENCES

Fig. 1. A portion of a cellulose acetate immunodiffusion strip showing radial precipitin patterns obtained by reacting anticonalbumin against known standards, serum obtained from chickens with TLT at 12 days postinoculation, and serum from noninoculated control chickens. S, standard; T, tumor; C, control.

Fig. 2. Lymphoid tumor cells infiltrating between skeletal muscle fibers at the site of implantation. H & E, X 160.

Fig. 3. Focal areas of hemorrhage (h) and necrosis (n) at the margin between TLT and viable tissue. H & E, X 160.

Fig. 4. Infiltration of liver sinusoids by lymphoid tumor cells 12 days after the implantation of TLT in the pectoral muscles. H & E, X 224.

Fig. 5. Autoradiogram of TLT cells 9 days after tumor implantation and 72 hr after intracardial injection of 59Fe-labeled transferrin. Hematoxylin, X 625.

Fig. 6. Higher magnification of Fig. 5 showing grains over the nucleus and cytoplasm. Hematoxylin, X 800.
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