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 $^{59}$Fe-labeled transferrin was used as a tracer. The transplantable lymphoid tumor incorporated $^{59}$Fe at a rate that was approximately 3 times that of the spleen and three-fourths that of the liver when $^{59}$Fe uptake was related to organ weight at 24 hr postinjection. Autoradiography at the light microscopic level showed that $^{59}$Fe 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$^3$ 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 $^{59}$Fe-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, unclotted (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.

Received July 31, 1972; accepted October 27, 1972.

1 Published as Manuscript 914. Supported in part by General Research Support Grant 10-03-RR-208-014 (67) from the NIH.
2 USPHS postdoctoral trainee in pathology.
3 The abbreviations used are: SI, serum iron; TLT, transplantable lymphoid tumor; TIBC, total iron-binding capacity; PCV, packed cell volume.

CANCER RESEARCH VOL. 33
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 4° overnight, and then the serum was removed. SI and TIBC were determined with a commercial kit (Ferro-check; 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 controls) of the 55 chickens studied. We used a 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 37° 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 Cl-; 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 nontumor control chickens, received intracardiac 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 Permoun (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 (X800) 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 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 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
Lymphoid Tumor Uptake of Radioiron

**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

<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>N.D.°</td>
<td>257.1 ± 15.9 (5)</td>
</tr>
<tr>
<td>Serial-bleeding control</td>
<td>200.0 ± 25.8 (3P)</td>
<td>222.5 ± 44.4 (3P)</td>
</tr>
<tr>
<td>Tumor</td>
<td>284.8 ± 16.2 (19)</td>
<td>351.6 ± 18.1 (14)</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>N.D.</td>
<td>142.5 ± 8.3 (4)</td>
</tr>
<tr>
<td>Serial-bleeding control</td>
<td>140.4 ± 14.7 (5)</td>
<td>125.0 ± 6.1 (5)</td>
</tr>
<tr>
<td>Tumor</td>
<td>242.5 ± 29.8 (5)</td>
<td>322.5 ± 37.8 (6)</td>
</tr>
</tbody>
</table>

* a N.D., not done.
* b Numbers in parentheses (unless otherwise footnoted), number of animals.
* c (3P), pooled serum samples of serial controls. There were at least 3 individual samples in each of the 3 pools.

TIBC: transferrin ratios calculated from the mean values (Table 1) of the control and TLT groups indicate that transferrin from chickens bearing TLT binds only about two-thirds as much iron as that from the noninoculated control groups. Statistical significance (p < 0.01) was found when the mean transferrin values of the 0-day and serial- and single-bleeding control groups were compared with those of the 7- and 12-day TLT groups.

Histological sections of TLT stained with hematoxylin and eosin revealed sheets of large lymphoid cells scattered between skeletal muscle fibers at the site of implantation (Fig. 2). These lymphoid cells were characterized by a high mitotic index, large vesicular nuclei with prominent nucleoli, and a scant basophilic cytoplasm. Slight to moderate amounts of hemorrhage and necrosis were usually confined to the margin between tumor and viable tissue (Fig. 3). The liver (Fig. 4) in some chickens contained numerous metastatic foci of TLT detectable by microscopic but not by gross examination. Tumor foci were not seen in the bone marrow. Staining with Perls’ reagent (for iron pigment) revealed little or no detectable hemosiderin in the tumor and bone marrow, but there was a moderate to marked increase of detectable hemosiderin in the spleen and liver of most but not all of the TLT chickens, compared with controls.

Chickens inoculated with TLT continued to eat and drink throughout the experiment. At necropsy, the crops were filled with food, and most of the chickens appeared well nourished.

**Trial 2**

**Distribution of ⁵⁹Fe in TLT.** The majority of the TLT cells were labeled at both 24 and 72 hr following the injection of radioiron (Fig. 5). No differences were noted in the degree of labeling between each time interval. Grains were almost entirely concentrated over tumor cells, and there was no appreciable labeling over blood vessels, erythrocytes, and necrotic or interstitial areas of the tumor. However, grains were located around the periphery of some degenerating skeletal muscle fibers intimately associated with the tumor. Labeling of TLT cells was not uniform throughout the autoradiograms. Some cells contained large amounts of radioiron, as indicated by the numerous grains over the cytoplasm and/or nucleus, whereas other cells exhibited few or no grains. The intracellular distribution of grains over TLT cells varied, with most cells showing predominantly cytoplasmic uptake while a few showed a combination of nuclear and cytoplasmic uptake (Fig. 6).

**Distribution of ⁵⁹Fe 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 ⁵⁹Fe.** The percentage of injected ⁵⁹Fe/g, wet weight, of tissue from tumor-bearing and nontumorous controls at 24 and 72 hr postinjection is shown in Table 2. The 24-hr bone marrow ⁵⁹Fe 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 ⁵⁹Fe/g of liver and spleen, while the uptake of ⁵⁹Fe/g of TLT remained relatively constant. One g of TLT accumulated approximately 15 times as much ⁵⁹Fe as did the bursa of Fabricius, a lymphoid organ with cells morphologically similar to those of the tumor. When ⁵⁹Fe uptake was related to organ weight at 24 hr postinjection, the entire TLT incorporated ⁵⁹Fe at a rate that was approximately 3 times that of the spleen and three-fourths that of the liver.

**DISCUSSION**

TLT cells of chickens inoculated radioiron from ⁵⁹Fe-labeled transferrin in vivo. Results of the tissue quantitation studies indicate that the TLT has a major role in plasma ⁵⁹Fe 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...
showed increased iron stores in the spleen and liver of most
was suggested, but not demonstrated, that iron incorporation
Burger and Lajtha (5) did not determine whether the heme-synthesizing capability of
chronic leukemia could not incorporate radioiron into heme.
suspension cultures. When iron was removed from the culture
chromosomes of HeLa S3 cells grown in monolayers and
neoplastic transformation. Walters et al. (36) found that
needed for rapid division of neoplastic cells was provided by
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
in vivo 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 59Fe by chicken TLT cells and a reduced
59Fe 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 59Fe 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 transplatable
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 ovitut was not a factor in these observations.

ACKNOWLEDGMENTS

The authors thank Dr. John M. Bowen for advice concerning
statistical interpretation and Mr. John-lin Chen for technical assistance.
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.
Radioiron Incorporation by a Transplantable Lymphoid Tumor

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


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/33/2/342

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/33/2/342.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.