Elevated Serum Iron, Low Unbound Transferrin and Candidiasis in Acute Leukemia

By LEONA CAROLINE, FRED ROSNER AND PHILIP J. KOZINN

TRANSFERRIN OR SIDEROPHILIN is the iron binding protein of human plasma and has the electrophoretic mobility of a beta-l-globulin. Normally only about one third of the transferrin is saturated with iron. In 1946, Schade and Caroline demonstrated the bacteriostatic effect of transferrin. This in vitro antimicrobial power is depressed by free iron and enhanced by an increase in iron-binding capacity. Fresh human serum has also been shown to be fungistatic. This inhibition of fungal growth can be overcome by the addition of iron. Thus, the fungistatic principle in normal human serum may be transferrin.

Serum from patients with acute leukemia has been shown to have a markedly reduced capacity to inhibit the growth of yeasts such as Candida. The present report demonstrates that many patients with acute myeloblastic leukemia have elevated serum iron levels and nearly totally saturated serum iron-binding capacities. Some of these patients had systemic candidiasis, elevated Candida agglutination titers and positive anticandidal precipitin tests. It is postulated that these aberrations in iron metabolism in patients with acute leukemia may be causally related to the development of candida and other fungal infections. In vivo and in vitro studies support this hypothesis.

MATERIALS AND METHODS

A. Clinical Studies

Patients with the following diseases were studied: acute myeloblastic leukemia (34 patients); acute lymphoblastic leukemia (6 patients); chronic lymphocytic leukemia (13 patients); chronic myelogenous leukemia (10 patients); polycythemia vera (12 patients); lymphoma (14 patients); multiple myeloma (11 patients). Unequivocal diagnoses were established in each patient on the basis of clinical, peripheral blood, and bone marrow findings and/or tissue sections and other laboratory determinations such as serum and urine protein analyses in patients with multiple myeloma and blood volume determinations.
patients with polycythemia vera.

Serum iron and iron binding capacity were measured by the method of Schade, Oyama, Reinhart and Miller. Candida agglutination titers were determined by a slight modification of the method of Thjotta, Jansen and Rasch. Precipitin bands against candidal antigen were measured by the immunodiffusion technic.

B. In Vitro Studies

Materials: Glassware—All glassware was rendered iron-free by soaking in nitric acid for 18 hours. Sterile demineralized water was used throughout.

Leukemic Sera—Serum from three patients with acute myeloblastic leukemia in whom the transferrin was 96 per cent saturated (one patient) or 100 per cent saturated (2 patients) were employed.

Normal Serum—A single healthy man was used as a source of normal serum. His serum iron was 100 µg per cent and his iron binding capacity 30 per cent saturated.

High Unbound Iron Binding Capacity Serum—Pooled serum obtained from patients with iron deficiency anemia was used. This serum had an iron level of 80 µg per cent and an unbound iron binding capacity of 286 µg per cent (23 per cent saturated).

Glucose—A 1 per cent solution was prepared, sterilized by filtration and the pH verified at neutrality with a glass electrode.

Ascorbic Acid—An aliquot of 12.5 mgm ascorbic acid was dissolved in 5 ml of iron free water immediately before use. This solution was then diluted with 9 parts of 1 per cent glucose. The addition of .06 ml of this ascorbate-glucose mixture to 0.5 ml of serum brought the final concentration of ascorbic acid to 0.0025 mgm per cent and that of glucose to 100 mgm per cent.

Human Transferrin—Iron-free, undenatured, purified human transferrin (Behringwerke, Germany) was obtained through the courtesy of Dr. A. L. Schade of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. Ten milligrams of this substance was dissolved in 1 ml of iron-free water. Thus 0.1 ml of solution contained 1 mgm transferrin.

Iron Standard—A stock solution containing 702 mg of (NH₄)₂Fe(SO₄)₂·6H₂O dissolved in 100 ml of 0.06 N HCl was made and 15 mgm of ascorbic acid added. This solution containing 1 mg iron per ml was stored at 4 C. A fresh 1:20 dilution was made immediately before use and this final working solution thus contained 50 µg iron per ml.

Candida Albicans Inoculum—An 18 hour culture of C. albicans Tyson grown at 37 C. on 4 per cent glucose, 1 per cent neopeptone agar (Difco) was used. This organism is a laboratory strain originally isolated from a patient with a positive blood culture and conforms to the cultural identification criteria of Candida albicans. A 10⁶ suspension of the organism was made in sterile, demineralized water and a chamber count was performed on an aliquot. The organism suspension was diluted to contain 5000 viable C. albicans per ml or 150 units in 0.03 ml.

METHOD OF Procedure

All sera were collected aseptically using iron-free glassware. The sera were stored at 20 C. until used. The various components of the test system were added to 13 X 100 mm. sterile pyrex test tubes according to the schema outlined in Table 1. All tests were performed in duplicate. Test tubes 1 and 2 were aimed at examining the growth of Candida albicans in serum from normal and leukemic patients respectively in the presence of glucose and ascorbic acid. Tube 3 tested the effect of exogenously added iron sufficient to saturate fully the endogenous transferrin in normal serum to determine whether the known fungistatic effect of normal serum could be overcome. Tube 4 combined leukemic serum (100 per cent saturation of transferrin) with a serum containing high unbound iron binding capacity to determine whether the expected growth of yeast in the leukemic serum could be overcome by the addition of unsaturated transferrin. Tube 5 was identical to tube 4 except exogenous iron was added to saturate fully the previously added high unbound iron binding capacity serum. Tube 6 determined the ability of exogenous purified human trans-
Table 1.—Experimental Schema for in vitro Studies. Each + represents the presence of the material indicated in the left hand column. Blank spaces indicate absence of these substances.

<table>
<thead>
<tr>
<th>CONTENTS of TUBES</th>
<th>TEST TUBE NUMBERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Serum (30% Saturation of transferrin)</td>
<td>1  2  3  4  5  6  7</td>
</tr>
<tr>
<td>Leukemic Serum (100% Saturation of Transferrin)</td>
<td></td>
</tr>
<tr>
<td>High U.I.B.C. Serum (23% Saturation of Transferrin)</td>
<td></td>
</tr>
<tr>
<td>Human Transferrin (1 mg)</td>
<td></td>
</tr>
<tr>
<td>Iron Standard (2.5 μg/m)</td>
<td>1</td>
</tr>
<tr>
<td>Ascorbic Acid (.0025 mg% final concentration)</td>
<td>1  2  3  4  5  6  7</td>
</tr>
<tr>
<td>Glucose (100 mg% final concentration)</td>
<td>1  2  3  4  5  6  7</td>
</tr>
<tr>
<td>Candido Albicans Inoculum (150 organisms)</td>
<td>1  2  3  4  5  6  7</td>
</tr>
</tbody>
</table>

* U.I.B.C. Unbound Iron Binding Capacity

All tubes are subjected to a stream of CO₂ in air for 1 minute and then incubated at 20°C for 48 hours.

Ferrin to reverse candidal growth in leukemic serum. Tube 7 was identical to tube 6 except iron was added to neutralize the effect of the added purified human transferrin. All tubes contained equal volumes of total contents at pH 7.3.

After the additions were made according to the schema outlined in Table 1, all the tubes were shaken by hand and equilibrated with a stream of 5 per cent CO₂ in air for one minute. Tubes were closed with sterile rubber stoppers and incubated at 20°C, for 48 hours. Wet mount examinations and viable plate counts were made at 24 and 48 hours as described below.

Viable Plate Counts—There was little filamentation or germ tube formation by the Candida albicans under all the conditions described. In some cases, small clumps of yeast cells were noted. In other tubes, large aggregates appeared, as if the yeast cells were not released following budding. A semi-quantitative estimation of the total yeast growth was made, using a uniform method of dilution and plating.

Serial dilutions were made in sterile demineralized water to yield approximately 100 colonies per plate. Aliquots of final dilution were measured into sterile petri dishes. Melted
Table 2.—Increased Serum Iron Levels and Complete Saturation of the Iron Binding Capacity in Patients with Leukemia, Polycythemia Vera, Lymphoma and Multiple Myeloma.

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of Patients</th>
<th>No. with High Serum Iron (&gt;120 μg%)</th>
<th>No. with Fully Saturated Transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Myeloblastic Leukemia</td>
<td>34</td>
<td>21**</td>
<td>17**</td>
</tr>
<tr>
<td>Acute Lymphoblastic Leukemia</td>
<td>6</td>
<td>2*</td>
<td>2*</td>
</tr>
<tr>
<td>Chronic Myelocytic Leukemia</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Chronic Lymphocytic Leukemia</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polycythemia Vera</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Multiple Myeloma</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* 1 Patient had Systemic Candidiasis  
** 2 Patients had Systemic Candidiasis

4 per cent glucose, 1 per cent neopeptone agar at 46 C. was added and the plate contents mixed well. Plates were allowed to harden, inverted and then incubated at 37 C. for 48 hours. The colonies were counted and appropriate calculations were made.

**RESULTS OF CLINICAL STUDIES (Table 2)**

A. Acute Leukemia

Twenty-one (64 per cent) of the thirty-four patients with acute myeloblastic leukemia in relapse had elevated serum iron levels (greater than 120 μg. per cent; normal range in this laboratory is 70–120). In seventeen of these twenty-one patients, the iron binding globulin was fully saturated (UIBC equals 0) on at least one occasion and usually repeatedly on several determinations. In an additional ten patients, the per cent saturation of transferrin was greater than 50 per cent. Normally, transferrin is only one third saturated (normal
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range of TIBC in this laboratory is 270–330 μg. per cent). Seven patients in the total group of acute myeloblastic leukemia patients had significant Candida agglutination titers (1:320 or greater) and two of these patients had proved systemic candidiasis including positive blood cultures. Both latter patients also had positive precipitin bands against candidal antigen and both were successfully treated with amphotericin B.

Two of six patients with acute lymphoblastic leukemia had elevated serum iron levels (226 μg. per cent and 149 μg. per cent) and very low or absent unbound transferrin (4 μg. per cent and 0 μg. per cent). One of these patients had systemic candidiasis proved at autopsy. In the other patient, both serum iron and unbound transferrin reverted to normal when clinical and hematologic remission was achieved. A third patient had normal serum iron and unsaturated transferrin values but developed systemic candidiasis with positive agglutination titers, precipitin tests and Candida cultures from throat, stool and urine.

B. Chronic Leukemia

In two of ten patients with chronic myelogenous leukemia, there were elevated serum iron levels (184 μg. per cent and 144 μg. per cent) and near or complete saturation of transferrin (96 per cent and 100 per cent respectively). Neither patient had clinical or laboratory evidence of candidiasis.

In none of the thirteen patients with chronic lymphocytic leukemia was the serum iron increased or the unbound transferrin level decreased. None of these patients had signs of candidal infection.

C. Other Disorders

None of the patients with multiple myeloma or polycythemia vera had abnormalities in serum iron or iron binding capacity. Among the fourteen patients with lymphoma, one patient with reticulum cell sarcoma and another with lymphosarcoma had increased serum iron levels (214 μg. per cent and 125 μg. per cent) and low or absent unbound transferrin values (30 μg. per cent and 0 μg. per cent respectively). Neither patient had clinical or laboratory evidence of candidal infection.

RESULTS OF IN VITRO STUDIES

Three separate but identical sets of experiments were conducted as described above, using sera from three patients with acute myeloblastic leukemia during relapse. In two of the patients, transferrin was completely saturated and in the third it was 96 per cent saturated. Similar results were obtained in all three instances (Table 3). Mean values for the three sets of experiments are graphically represented and summarized in Figure 1.

Growth of Candida albicans in the leukemic sera was approximately 1000 times greater than in normal serum. This marked enhancement of growth occurred when only 150 units of Candida albicans were inoculated into each serum and incubated at 20 C. for 48 hours. Addition of iron sufficient to saturate fully the transferrin in normal serum permitted growth of Candida albicans comparable to that in serum of the leukemic patients. (Table 3 and
Table 3.—Growth of Candida Albicans in Serum from Normal and Leukemic Subjects.

<table>
<thead>
<tr>
<th>Test Mixtures</th>
<th>Patient's % Saturation of Transferrin</th>
<th>Viable Units of Candida Albicans per ml after 48 hours incubation at 20°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Normal Serum (30% saturation of transferrin)</td>
<td></td>
<td>36,500 16,900 17,675</td>
</tr>
<tr>
<td>2) Leukemic Serum (96% or 100% saturation of transferrin)</td>
<td></td>
<td>36,662,500 35,600,000 4,563,000</td>
</tr>
<tr>
<td>3) Normal Serum Plus Iron to fully saturate the U.I.B.C.*</td>
<td></td>
<td>28,562,500 26,300,000 34,125,000</td>
</tr>
<tr>
<td>4) Leukemic Serum Plus high U.I.B.C.* Serum</td>
<td></td>
<td>37,500 40,400 3,000</td>
</tr>
<tr>
<td>5) Leukemic Serum Plus high U.I.B.C.* serum plus iron to fully saturate the U.I.B.C.*</td>
<td></td>
<td>25,050,000 66,600,000 9,450,000</td>
</tr>
<tr>
<td>6) Leukemic Serum plus Purified Human Transferrin</td>
<td></td>
<td>49,500 21,750 12,000</td>
</tr>
<tr>
<td>7) Leukemic Serum Plus Purified Human Transferrin Plus Iron to Fully saturate the transferrin</td>
<td></td>
<td>31,637,500 78,500,000 16,500,000</td>
</tr>
</tbody>
</table>

* U.I.B.C. = unbound iron binding capacity

Fig. 1.—Growth of Candida Albicans in serum from normal and leukemic subjects. Mean values of three sets of experiments using sera from three leukemic patients.
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Fig. 1). When, however, fully transferrin saturated serum from leukemic patients was combined with a highly transferrin unsaturated serum (obtained from a patient with iron deficiency anemia), candidal growth was inhibited. If to this mixture of serum from leukemic patients and highly transferrin unsaturated serum, sufficient iron was added to fully saturate the unbound iron binding capacity, the protection against candidal growth afforded by the unbound binding capacity was neutralized.

Adding an exogenous source of purified human transferrin to fully transferrin saturated serum from leukemic patients inhibited growth of Candida albicans. This inhibition of growth was overcome by the addition of sufficient iron to saturate fully the added transferrin. Leukemic serum whose iron saturation was within normal limits failed to support fungus growth.

DISCUSSION

In 1944 Schade and Caroline described a protein component in raw egg white that was capable of binding iron and inhibiting growth of Shigella dysenteriae and other microorganisms. This inhibition of growth could be overcome only by the addition of iron but not by ten vitamins or 30 other elements tested. Two years later, Schade and Caroline demonstrated a bacteriostatic iron binding protein in human plasma and called it siderophilin (now called transferrin or iron-binding globulin). Other antimicrobial substances in human serum have also been described and these include gram negative bactericidins, beta lysins and properdin.

Fresh human serum has also been shown to contain a potent factor which inhibits the growth of certain fungi. Serum from normal adults possesses a high capacity to inhibit growth of Candida albicans, an effect which can be overcome by the addition of iron. This inhibitory activity is greatly diminished in umbilical cord blood and infant blood up to 10 weeks of age. The low resistance of the newborn to candidiasis may be due to the fact that the iron binding capacity of cord blood and newborn blood is nearly fully saturated with iron.

One can postulate a similar mechanism to explain the increased susceptibility of patients with acute leukemia to fungus infections. The high serum iron and/or nearly complete or full saturation of transferrin in the serum of such patients may foster candidal growth. Ionic iron is important in determining whether infectious agents can multiply in mammalian host tissues. Yeasts such as Candida albicans can grow in the presence of normal serum or plasma only when sufficient iron is available to saturate fully the transferrin. Jackson and Morris have shown that the virulence of Pasteurella and other bacteria is enhanced by the addition of iron. Martin, Jandl and Finland demonstrated the enhancement of virulence of Klebsiella pneumoniae and Pseudomonas aeruginosa in rats and mice when iron salts were administered. Small doses of iron-free human transferrin afforded some degree of protection against acute infections with these organisms. Iron is also essential for the growth of Clostridium welchii, Listeria monocytogenes, staphylococcus and salmonella. Cations such as magnesium, calcium, cobalt, nickel, copper, zinc and others cannot substitute for iron.
Roth and Goldstein have shown that the serum of normal adults possesses marked capacity to inhibit the in vitro reproduction of Candida albicans and other species of Candida. In patients with acute leukemia, they found the activity or concentration of the inhibitory complex to be greatly diminished. These investigators did not, however, measure serum iron or iron binding capacity in their patients. In a study of eight patients with Hodgkin's disease, Cline and Berlin found an increased serum iron concentration in one patient on one occasion and a low value in the same patient on another occasion. The iron binding capacity was fully saturated in another of these patients on one of three occasions. Neither patient is reported to have developed candidal infection.

The hypothesis that the increased frequency of systemic fungal infections, particularly candidiasis, in patients with acute leukemia is causally related to the high iron and/or near complete or full saturation of transferrin in the serum of such patients is supported by clinical observations and in vitro experiments. Twenty-one of thirty-four patients with acute myeloblastic leukemia (64 per cent) and two of six patients with acute lymphoblastic leukemia had elevated serum iron levels and very low or absent unsaturated transferrin levels. Whether the iron binding globulin itself protects an individual by directly inhibiting fungal growth or whether the excess iron in the plasma enhances candidal growth has not yet been determined. These two factors are difficult to study separately because of the high avidity for iron of the unsaturated iron binding capacity of human serum. In this manner, inhibition of multiplication of fungi and bacteria is achieved by depriving these microorganisms of biologically available iron essential for their reproduction.

Leukocytes contain appreciable amounts of iron and the state and physiologic function of this iron has not yet been determined. Leukocytes destroyed by the invading organisms, or leukemic cells destroyed by chemotherapeutic agents may release iron that is biologically available to microorganisms for multiplication. Another possible source of iron in the patient is blood transfusion from which hemolysed erythrocytes release iron into the circulation. However, in the studies here reported several patients had high serum iron levels and low or absent unbound iron binding capacities before any blood had been administered. The possibility that the patient was destroying his own red cells at an accelerated rate with release of excess iron into the blood was not investigated.

Other possible mechanisms may explain the increased incidence of fungus infections in patients with acute leukemia. One proposed explanation is that such patients are usually receiving one or more antibiotics, possibly predisposing them to the development of superinfections. Antibiotics may increase the hazard of candidiasis by changing the intestinal flora, thereby permitting overgrowth of Candida in the absence of competing organisms. Other mechanisms may include local tissue damage and increased invasiveness of the Candida. In addition, antibiotics inhibit anticandidal antibody synthesis and phagocytic activity, thus reducing host resistance to invasion by Candida.
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The role of corticosteroids and antileukemic drugs in the sharp increase in mycotic infections in leukemic patients in the last two decades, cannot be minimized.\textsuperscript{26,27} Host resistance in leukemic patients with already poor immunologic defenses may be further depressed by corticosteroids and antileukemic drugs. However, in seven of the patients with acute myeloblastic leukemia here reported who had fully saturated iron binding capacities, no specific antileukemic chemotherapy had yet been administered at the time they were studied. Furthermore, preliminary studies in patients with Cooley's anemia who are not receiving such chemotherapeutic agents, reveals enhanced candidal growth in their serum when compared to normal individuals. Patients with Cooley's anemia have low or absent unsaturated transferrin and elevated iron levels in the serum.\textsuperscript{28}

Immunologic competence in patients with acute leukemia is not totally lacking in that host defenses against fungi may remain intact. A specific host antibody response to \textit{Candida albicans} is the production of precipitating antibodies specific for a somatic antigen\textsuperscript{14} of this fungus in the serum of the patients who develop systemic candidiasis. The specific component in the serum of such patients that reacts with the somatic antigen of \textit{Candida} is presently being studied. Immunoelectrophoretic investigations seem to indicate that this material is distinct from the defined human immunoglobulins.

Further questions remain to be answered. Do patients with iron deficiency anemia who have high levels of unsaturated iron binding capacity develop fewer fungal infections than normal individuals? Would the intravenous administration of purified human transferrin protect patients with acute leukemia against fungal infections? Why do children with Cooley's anemia who have fully saturated transferrin in their plasma not develop systemic candidiasis as do patients with acute leukemia? What is the significance of the reduced levels of iron binding capacity present in patients with acute and chronic bacterial infections? Are these two findings causally related? Do patients with hemochromatosis or other forms of iron overload develop more infections than normal people? What effect, if any, does antifungal chemotherapy (i.e. amphotericin B) have on plasma transferrin levels and per cent saturation?

**SUMMARY**

Patients with acute leukemia have been shown to have elevated serum iron levels and markedly diminished or absent unbound iron binding capacity (21 of 34 patients with myeloblastic and 2 of 6 patients with lymphoblastic leukemia). Four patients (2 myeloblastic and 2 lymphoblastic) developed systemic candidiasis with elevated \textit{Candida} agglutination titers and positive precipitin tests against a somatic antigen of \textit{Candida albicans}. It is postulated that high serum iron and/or low unbound transferrin predispose patients with acute leukemia to candidal infection by enhancing growth of this fungus. In vitro studies support this hypothesis.

**SUMMARIO IN INTERLINGUA**

Ha essite monstrate que patientes con leucemia acute ha elevate nivellos de ferro seral e un marcatemente reducite o absente capacitare de ligar ferro non ligate (21 de 34...
patients con leucemia myeloblastic e 2 de 6 pacientes con leucemia lymphoblastic). Quatro pacientes (2 myeloblastic e 2 lymphoblastic) desenvolvavam candidiasis con elevate titros de agglutination a *Candida* e positive tests de precipitina contra un antigeno somatic de *Candida albicans*. Es postulate que alte concentrationes de ferro seral e/o basse concentrationes de transferrina non-ligate patientes con leucemia acute a infection per *Candida* in consequentia de lor effecto promotori super le crescentia de iste fungo. Iste hypothese es supportate per studios effectuate in vitro.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Arthur L. Schade for helpful advice and encouragement and to Mrs. Claire L. Taschdjian and Mr. Manuel Cuesta for performing the Candida agglutination and precipitin tests. Thanks are due to Mrs. Kay D. Orr for technical assistance and to Miss Gail Wollan for typing the manuscript.

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