The Effect of Chelating Agents on Iron Mobilization in Chang Cell Cultures

By G. P. White, A. Jacobs, R. W. Grady, and A. Cerami

The investigation of chelating agents with potential therapeutic value in patients with transfusional iron overload has been facilitated by the use of Chang cell cultures. These cells have been incubated with [^{59}Fe]transferrin for 22 hr, following which most of the intracellular radioiron is found in the cytosol, distributed between a ferritin and a nonferritin form. Iron release from the cells depends on transferrin saturation in the medium, but when transferrin is 100% saturated, which normally does not allow iron release, desferrioxamine, 2,3-dihydroxybenzoic acid, rhodotorulic acid, cholylhydroxamic acid, and tropolone all promote the mobilization of ferritin iron and its release from cells. They are effective to an approximately equal degree. The incubation of [^{59}Fe]transferrin with tropolone in vitro at a molar ratio of 1:500 results in the transfer of most of the labeled iron to the chelator, reflecting the exceptionally high binding constant of this compound. How far these phenomena relate to therapeutic potentiality remains to be seen.

IN PATIENTS WHO HAVE REPEATED BLOOD TRANSFUSIONS, gradual accumulation of iron results from the lack of a physiologic mechanism for iron excretion. The widespread iron deposition and tissue damage leads to eventual death, usually from myocardial or hepatic failure. The majority of affected patients are children or adolescents with homozygous β-thalassemia who have been treated with a high transfusion regime or subjects with chronic hypoplastic anemia. Relatively little is known about intracellular iron metabolism, and attempts to remove excess iron by the use of chelating agents have not been wholly successful. Recent studies have set out to clarify our knowledge of intracellular iron metabolism and help in providing a rationale for chelation therapy.

When cellular iron uptake is in excess of normal metabolic requirements, it results in the stimulation of ferritin synthesis[1] and in conditions of iron overload; some cells produce greatly increased amounts of ferritin. White et al.[2] have shown that Chang cells cultured in vitro provide a stable model system for the investigation of iron metabolism, and this has been used to study the effect of a number of chelating agents on iron uptake, ferritin synthesis, and the incorporation of iron both into ferritin and nonferritin pools within the cell sap.[3] The present study makes use of the same experimental system to examine the effect of chelating agents on iron release from Chang cells.

MATERIALS AND METHODS

Incubation of Cells with [^{59}Fe]Transferrin and [^{3}H]Leucine

Chang cells (Flow Laboratories, Ltd.) were cultured in Minimum Essential Medium (MEM) containing 10% fetal bovine serum. The cells were harvested after 10 days and washed with fresh

From the Department of Haematology, Welsh National School of Medicine, Cardiff, Wales, and the Department of Medical Biochemistry, The Rockefeller University, New York, N.Y.

Submitted June 29, 1976; accepted August 13, 1976.

Address for reprint requests: Dr. Allan Jacobs, Department of Haematology, Welsh National School of Medicine, University Hospital of Wales, Heath Park, Cardiff CF4 4XN, Wales.

medium. Serum from patients with iron deficiency was labeled with ^{59}Fe by the method of Cavill.[4] The saturation of the transferrin was determined by preliminary estimation of the iron concentration and total iron-binding capacity using Young and Hicks' method.[5] Calculated amounts of iron were added as [^{59}Fe]ferric citrate (Radiochemical Centre, Amersham), and ferric ammonium citrate to give a final specific activity of 112 μCi/μmole Fe.

Cells (4×10^7) were incubated in a mixture of MEM (4 ml) and labeled serum (4 ml) with the addition of 5 μCi [^3H]leucine (5 nmole) for 22 hr at 37°C under 5% CO_2 in air. Following incubation, the cells were washed five times with 5-ml portions of cold MEM and then resuspended in fresh MEM at a concentration of 3.3×10^6 cells/ml. Six tenths of a milliliter of serum (100% saturated unless otherwise stated) were incubated with 0.6 ml of cell suspension with the addition of various chelators (50 μl) for 6 hr at 37°C under 5% CO_2 in air. After incubation, the cells were washed three times with 5-ml portions of cold MEM, and then with two further washings of isotonic saline before sonication in 5 ml of saline. The volume was adjusted to 10 ml, and a 1-ml aliquot of the resulting suspension was taken for measurement of ^{59}Fe and [^3H]leucine incorporation into the cells. The remainder was centrifuged at 20,000 *g* for 40 min at 4°C, the supernatant removed, and a 1-ml aliquot taken for measurement of ^{59}Fe activity in the supernatant; 100 μg human spleen ferritin was added to 7 ml of the remaining supernatant, and the mixture was incubated with antihuman ferritin serum (50 μl) at 37°C for 1 hr. After sitting at 4°C overnight, the precipitate was washed three times with saline and dissolved in 1 ml 0.01 *M* HCl. The radioactivity of the three 1-ml fractions (cells, cell supernatant, and ferritin) was counted in an Intertechnique Liquid Scintillation Counter in 10 ml of scintillation cocktail (toluene/Triton × 100/PPO/POPOP; 700 ml/350 ml/3.5g/70 mg). The amount of activity in the nonferritin fraction of the cell supernatant was calculated as the difference between the supernatant fraction and the ferritin fraction. Iron and leucine uptake were measured by the method used previously.[3]

Chelating Agents

Chemicals of greater than 97% purity were obtained from the following sources and used without further purification: 2,3-dihydroxybenzoic acid and tropolone (Aldrich Chemical Company, Inc., Cedar Knolls, N.J.); desferrioxamine (Ciba Pharmaceutical Company, Summit, N.J.). Cholylhydroxamic acid was synthesized according to the method of Shimizu et al.[6] Rhodotorulic acid was

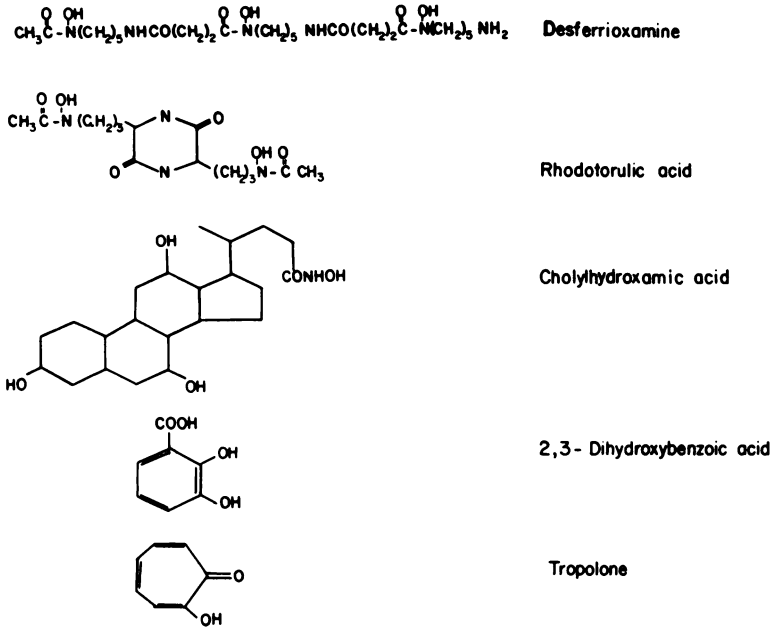

Fig. 1. Structures of the five chelators under study.

SUMMARY OF MEMBERSHIP[1]

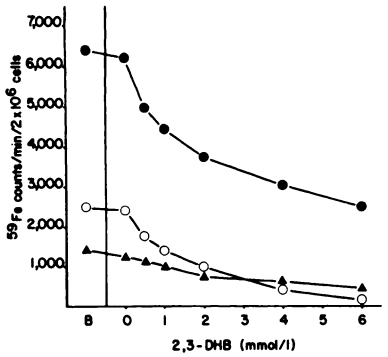

Fig. 3. ^{59}Fe activity in Chang cells after 6 hr of incubation with increasing concentrations of 2,3-DHB. Symbols as in Fig. 2.

original value. 2,3-dihydroxybenzoic acid that had been preincubated with rat liver homogenate also promoted iron release (Fig. 3), but the concentration producing 50% loss was four times greater than the corresponding concentration of desferrioxamine. Here too, a greater amount of iron was lost from ferritin than from the nonferritin fraction.

Rhodotorulic acid, cholylhydroxamic acid, and tropolone were also effective in removing labeled iron from Chang cells under these experimental conditions (Table 1). Tropolone was particularly effective at low concentrations, 40% of the iron being removed from ferritin with a concentration of 0.5 mmole/liter. Further increase in concentration resulted in only a small increase in the amount of iron removed, and concentrations above 1 mmole/liter were found to have a marked inhibitory effect on total protein synthesis. Gel chromatog-

Table 1. Percentage ^{59}Fe Removed from Chang Cells During 6 hr of Incubation at 37°C

Compound	(mmole/liter)	^{59}Fe Removed (%)	
		Whole Cells	Ferritin
Desferrioxamine	0.05	1	8
	0.10	13	25
	0.50	42	57
	1.00	47	61
2,3-DHB (after incubation with liver)	0.50	23	29
	1.00	30	44
	2.00	41	59
	6.00	61	94
Rhodotorulic acid	0.50	16	18
	1.00	25	34
	2.00	29	44
	6.00	38	58
Cholylhydroxamic acid	0.50	17	42
	1.00	22	46
	2.00	25	43
Tropolone	0.50	40	37
	1.00	42	40
	2.00	46	42
	6.00	54	57
Medium alone	—	2	5

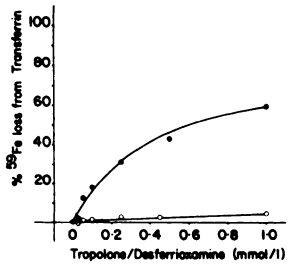

Fig. 4. ^{59}Fe loss from labeled transferrin after mixing with increasing concentrations of tropolone (●——●) or desferrioxamine (○——○).

raphy of the ^{59}Fe-containing cytosol using Sephadex G-200[2] before and after incubation with the various chelating agents showed that in all cases the ^{59}Fe chelate was derived from the nonferritin fraction and that there was no direct chelation of ferritin iron.

When the labeled cells were incubated with 0.5 mmole/liter desferrioxamine and fully saturated transferrin, 49% of the iron was released in 6 hr compared with 42% released on incubation with iron-deficient serum. When the cells were incubated with a combination of iron-deficient serum, together with 0.5 mmole/liter desferrioxamine, there was still only 47% iron release in 6 hr. When labeled cells were incubated with desferrioxamine (0.1 mmole/liter) and rhodotorulic acid (1 mmole/liter), the amount of iron released did not suggest a mutually potentiating effect when compared with the two chelators used separately. Similarly, there was only an additive effect when desferrioxamine was used together with 2,3-DHB.

The addition of 1 mmole/liter ascorbic acid to the medium had no effect on iron release from the cells, either in the presence of 100% saturated transferrin alone or when 0.5 mmole/liter desferrioxamine was present.

A mixture of 2 ml MEM and 100 μl of serum containing ^{59}Fe-labeled transferrin was dialyzed for 6 hr against a number of chelators at a concentration of 6 mmole/liter. Loss of ^{59}Fe activity amounted to 3% in the case of desferrioxamine, 1% for 2,3-DHB, 0% for rhodotorulic acid, and 84% for tropolone. A comparison of desferrioxamine and tropolone in their ability to remove iron from transferrin is shown in Fig. 4.

Postincubation of Chang cells for 72 hr following their labeling with [^{3}H]leucine showed the half-time for total protein turnover to be 58 hr and for ferritin turnover 110 hr. These estimates were essentially unchanged when the cells were incubated with 6 mmole/liter desferrioxamine, which removed most of the labeled iron from ferritin.

DISCUSSION

Previous studies have shown that Chang cells can be used to study cellular iron metabolism and that chelating agents can inhibit the accumulation of iron in cells incubated with [^{59}Fe]transferrin.[2,3] This inhibition is associated with a reduction in ferritin synthesis. White et al.[2] have demonstrated the existence of a low molecular weight, chelatable iron pool in these cells and the interaction of chelators with this iron prevents its stimulation of ferritin synthesis and, presumably, promotes its exit from the cell. None of the iron chelators previously studied removes iron directly from transferrin, except in minimal amounts. This

cell culture system has been used to screen a large number of iron-chelating agents of potential therapeutic value,[3] and, in the present study, the most effective agents have been used in the same in vitro system to examine their influence on iron mobilization. In addition, we have studied tropolone, a seven-carbon ring compound, which is also a known iron chelator.

Under normal incubation conditions, the rate of iron mobilization from the cell is dependent on the transferrin saturation in the medium (Fig. 2). Very little radioactive iron is lost when there is full saturation and few binding sites are available; what little there is probably is part of the normal flux between cell and medium. However, when the extracellular transferrin has a high latent binding capacity, up to 50% of the tracer may be lost during a 6-hr incubation. There is a considerable reduction in the amount of ferritin iron in the cytosol and some reduction of membrane-bound iron. The amount of nonferritin iron remains almost unchanged.

All experiments with chelators were carried out with fully saturated transferrin in the medium. The pattern of iron mobilization with increasing concentrations of chelator in the medium was similar to that seen when transferrin with an increasing latent binding capacity was used, though in this instance the exit of iron from the cell probably followed its intracellular chelation. White et al.[2] showed that ferritin iron in the cytosol was not easily available for chelation, while the nonferritin component bound readily to most chelating agents and to transferrin. It has been suggested that there is an equilibrium between ferritin iron and the labile iron pool, and that the mobilization of ferritin iron is secondary to mobilization of the labile iron. In the case of desferrioxamine and rhodotorulic acid, gel filtration of the cytosol after incubation of the cells with the chelator showed that much of the nonferritin iron was then present as the specific chelate.[2] At a desferrioxamine concentration of 0.5 mmole/liter, about 95% of the total cytosol iron was present as ferrioxamine after a 22-hr incubation. 2,3-DHB forms a potent chelating agent of unknown composition after incubation with liver homogenate,[2] and the effect of the resulting supernatant on iron release from the cells is shown in Fig. 3. It is unlikely that any of the compounds studied here would reach a concentration much greater than 1 mmole/liter in vivo, and, at this concentration, they were all effective in reducing both the total cell ^{59}Fe and the ferritin-^{59}Fe concentrations. None, however, was superior to desferrioxamine. It is of interest that the turnover time of labeled ferritin protein appeared unchanged when the labeled ferritin iron had been almost entirely removed by desferrioxamine. This finding suggested that iron-poor and iron-rich ferritin may have a similar stability and prompts reassessment of the view that iron plays a role in stabilizing the ferritin protein shell.

It has been suggested that different chelators react with different iron pools, so that two compounds together in equivalent amounts will chelate more iron than when either is used at twice the basic concentration.[7] The current experiments do not bear this out; when desferrioxamine is combined with either rhodotorulic acid or 2,3-DHB, the effect is similar to simply doubling the concentration of either compound. Similarly, no more iron is released from the cells by the presence of unsaturated transferrin and desferrioxamine together than

when they are present separately. While most of the iron chelators in current