Chromosome-damaging Activity of Ferritin and Its Relation to Chelation and Reduction of Iron\footnote{This work was supported by the National Cancer Institute of Canada and the Natural Sciences and Engineering Research Council of Canada.}

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ABSTRACT

Ferritin from horse spleen was found to cause severe chromosome aberrations in cultured Chinese hamster ovary cells. Ferritin at 15 to 170 \( \mu \)g/ml was clastogenic and at higher doses was cytotoxic. At comparable concentrations of protein or iron, neither apoferritin nor complexed iron was clastogenic. Thiol compounds glutathione and cysteine reduced the cytotoxic and clastogenic activities of ferritin. Physiological concentrations of glutathione may normally be sufficient to protect cells from damage. The reducing agent ascorbate had little protective effect. Chelating agents varied in their inhibitory activity: ethylenediaminetetraacetic acid (hexadentate) > nitrilotriacetic acid (tetradentate) > salicylate (bidentate). 2,2'-Bipyridyl enhanced the chromosome-damaging action of ferritin while histidine did not markedly alter the frequencies of aberrations. Catalase and superoxide dismutase showed no inhibitory activity. The mechanism of DNA damage may involve reduction of Fe(III) in the ferritin core to Fe(II), followed by reoxidation of Fe(II) with possible formation of free radicals.

INTRODUCTION

Ferritin is a major iron storage protein and is found widely distributed in animals, plants, and fungi (6, 9, 21). In humans, about 25% of the total body iron is contained in ferritin, stored mainly in liver, spleen, and bone marrow (6, 9, 21). During tumorigenesis, the amount of ferritin in tissues and serum may significantly increase, and ferritin with different electrophoretic mobilities was formed (23, 24). The ferritin molecule consists of a central iron core surrounded by a spherical protein shell called apoferritin. The iron core is a complex polymer of Fe(III) in the form of hydrous ferric oxide phosphate (6, 9, 21). Ferritin has been reconstructed from apoferritin, Fe(II), and an oxidant (2, 3, 10, 16, 29). Conversely, the reduction of Fe(III) to Fe(II) is involved in the release of iron from ferritin (4, 5, 11, 14, 20, 22, 25, 27, 30).

Iron:carbohydrate complexes are carcinogenic (12), and ferric sulfate is mutagenic in bacteria (1). In addition, low levels of ferritin (0.25 to 5.0 \( \mu \)g/ml culture medium) were found to suppress mitogen (phytohemagglutinin or concanavalin A)-induced blastogenesis in human peripheral blood lymphocytes (19).

We have been investigating the mutagenic and comutagenic activity of iron compounds (28, 31, 32). The interaction of iron complexes with sodium ascorbate caused high frequencies of chromosome aberrations in CHO\footnote{To whom requests for reprints should be addressed.} cells (28). Ferritin was of interest as a major physiological form of iron. In this report, we describe (a) the induction of chromosome damage by ferritin in cultured mammalian cells, (b) the effect of chelating and reducing agents on the clastogenic activity of ferritin, and (c) a comparison of the chromosome-damaging activity of ferritin with that of complexes of Fe(II) and Fe(III).

MATERIALS AND METHODS

Cell Cultures. CHO cells were grown in MEM (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% fetal calf serum, antibiotics (streptomycin sulfate, 29.6 \( \mu \)g/ml; penicillin G, 204 \( \mu \)g/ml; kanamycin, 100 \( \mu \)g/ml; and Fungizone, 2.5 \( \mu \)g/ml), and 7.5% sodium bicarbonate (10 ml/800 ml medium). The stock cultures were maintained in 250-ml plastic culture flasks (Falcon Plastics, div. Becton, Dickinson and Co., Cockeysville, Md.) at 37\(^\circ\) in a water-saturated \( CO_2 \) incubator.

For each chromosome aberration experiment, approximately 140,000 CHO cells were seeded on 22-sq mm coverslips in 3.5-cm plastic dishes (Falcon) and kept in MEM with 15% fetal calf serum at 37\(^\circ\) for 2 days. Experiments were begun when cells were 60 to 70% confluent.

Chemicals. The following chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.: ferritin [from horse spleen as sterile solutions in 0.15 M NaCl, different lots containing 84 to 100 mg of ferritin per ml, and 144 to 188 \( \mu \)g of iron per mg of ferritin (determined by atomic absorption by Sigma)]; apoferritin (from horse spleen as a sterile solution in 0.15 M NaCl, containing 50 mg of protein per ml); glutathione (reduced form, 98 to 100%); cysteine (HCl hydrate); histidine (free base); NTA (disodium salt, 99.5%); catalase (from bovine liver, with activity of 10,000 to 25,000 Sigma units per mg of protein); superoxide dismutase (from bovine blood, with approximately 3,000 units per mg of protein); FMN (sodium salt, 95 to 97%); \( \beta \)-NADH (disodium salt, 98%); ascorbic acid (sodium salt); salicylic acid; ferrous sulfate (heptahydrate, 99%); ferric chloride (hexahydrate); EDTA (disodium salt, dihydrate, 99%); and 2,2'-bipyridyl.

Iron Complexes. Complexes of Fe(II) and Fe(III) were freshly
preparing and diluted just before addition to the cultured cells. Stock solutions of FeSO₄·7H₂O and FeCl₃·6H₂O were prepared in double-distilled H₂O at concentrations of 0.1 M. Likewise, stock solutions of complexing agents were prepared in H₂O at the following concentrations: glycine and cysteine, 0.5 M; histidine, NTA, and EDTA, 0.2 M. The stock solutions were combined to give molar ratios of chelating agent to iron of 10:1 (glycine, histidine, and cysteine) or 2:1 (NTA and EDTA). The complexes were diluted as required into 2.5% MEM (MEM with 2.5% fetal calf serum) before addition to cells.

**Treatment of Cells with Chemicals.** All chemicals were freshly prepared immediately before addition to cells. Catalase, superoxide dismutase, or FMN was dissolved in ice-cold 2.5% MEM. All other chemicals were dissolved and diluted in 2.5% MEM at room temperature.

Equal volumes (0.8 ml) of ferritin solutions were mixed with the reducing or chelating agents directly on the cultured cells. The order of addition was (a) the reducing agent, the chelating agent, or the FMN:NADH system and (b) ferritin. The cells were incubated with the mixture for 3 hr at 37°C, washed with MEM, and incubated in 2 ml of 15% MEM for an additional 20 hr.

**Analysis of Chromosome Aberrations.** Cells were harvested 20 hr after the completion of chemical treatment. At 4 hr prior to harvesting, colchicine was added (final concentration, 10 µg/ml). Cells were then treated with hypotonic solution (1% sodium citrate) for 20 min, fixed in methanol:acetic acid (3:1, v/v) for 30 min, and air dried. The slides were stained with 2% orcein (in 50% acetic acid:water), dehydrated, and mounted. For each sample, 50 to 300 metaphase cells were analyzed for chromosome- and chromatid-type breaks and exchanges; gaps were not scored. Whenever possible, at least 100 metaphase cells were scored.

Chromosome aberration data in the tables are presented so as to provide 2 major measures of damage. The first entry is the frequency (percentage) of metaphase cells containing at least one break or exchange. The second entry (in parentheses) gives the average number of exchanges per cell, expressed as percentage.

## RESULTS

**Ferritin and Apoferritin.** The chromosome-damaging activity of ferritin and its corresponding iron-free protein shell, apoferritin, were examined. Ferritin (3 to 2760 µg/ml) or apoferritin (2 to 2240 µg/ml) in culture medium was incubated for 3 hr with CHO cells. The frequencies (percentage) of metaphase cells with chromosome aberrations and the average number of chromosome- or chromatid-type exchanges per cell are shown in Table 1.

Extensive chromosome aberrations were induced in CHO cells by ferritin at concentrations of 27 to 138 µg/ml. The frequency of cells containing aberrations reached 60%, while the number of exchanges per metaphase was also very high (up to 87%). Higher concentrations of ferritin (276 to 2760 µg/ml) inhibited mitosis and were cytotoxic. Low concentrations of ferritin (3 and 8 µg/ml) caused no detectable chromosome damage (cf. Fig. 1).

In contrast, apoferritin did not induce chromosome aberrations over a wide range of concentrations, i.e., 2 to 1120 µg/ml which is equivalent to the protein concentrations in 3 to 1380 µg of ferritin per ml. A marginal increase in aberrations (to 6.5%) was observed in cells treated with the highest concentration of apoferritin (2240 µg/ml, equivalent to the protein concentration in 2760 µg of ferritin per ml).

These results clearly indicate that the chromosome-damaging activity of ferritin is not associated with apoferritin, the spherical protein shell of ferritin.

**Ferritin and Iron Complexes.** In Table 2, the chromosome-damaging activity of ferritin and various complexes of Fe(II) and Fe(III) is compared. The chromosome aberrations induced by ferritin are expressed in relation to the iron content of the ferritin and of the iron complexes (the calculation was based on ferritin containing 18.8% iron). High concentration of ferritin (298 to 1490 µg/ml, equivalent to 10⁻³ to 5 x 10⁻³ M iron) was cytotoxic. Ferritin at lower concentrations (30 to 149 µg/ml, equivalent to 10⁻⁴ to 5 x 10⁻⁴ M iron) caused high frequencies of breaks and exchanges (Figs. 2 and 4), while ferritin at 3 µg/ml was not detectably clastogenic.

In comparison to the severe clastogenic effects of ferritin, iron complexes caused much less chromosome damage and were less toxic. Complexes of Fe(II) and Fe(III) were prepared with glycine, NTA, histidine, EDTA, and cysteine. At iron concentrations up to 5 x 10⁻⁴ M, none of the complexes caused increased frequencies of chromosome aberrations above those in nontreated control cells (cf. 69% aberrations with ferritin at 5 x 10⁻⁴ M iron). Moreover, the cells treated with glycine or histidine complexes of iron [as Fe(II) or Fe(III) at 1:10: amino acid molar ratio] showed background levels of aberrations. Fe(III):histidine at 3 to 5 x 10⁻³ M and Fe(II):histidine at 5 x 10⁻³ M caused cell loss or mitotic inhibition.

Chromosome aberrations were detected only in cells treated with mM concentrations of ferrous sulfate or iron complexes of NTA, EDTA, or cysteine. The NTA:iron or EDTA:iron molar ratio was 2:1 and the cysteine:iron molar ratio was 10:1. An increased frequency of chromatid breaks (5.6%) was induced by
Table 2
Frequency of chromosome aberrations in CHO cells treated with ferritin and iron complexes

<table>
<thead>
<tr>
<th>Ferritin concentration (µg/ml)</th>
<th>Chromosome aberrations (M)</th>
<th>Fe(II) complexes</th>
<th>Fe(III) complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1490</td>
<td>Toxic</td>
<td>5 x 10^{-3}</td>
<td>5.6 (0.0)</td>
</tr>
<tr>
<td>894</td>
<td>Toxic</td>
<td>3 x 10^{-3}</td>
<td>1.5 (0.0)</td>
</tr>
<tr>
<td>596</td>
<td>MI</td>
<td>2 x 10^{-3}</td>
<td>0.9 (0.0)</td>
</tr>
<tr>
<td>298</td>
<td>MI</td>
<td>1 x 10^{-3}</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>149</td>
<td>69.1 (97.5)</td>
<td>5 x 10^{-4}</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>59</td>
<td>29.2 (42.3)</td>
<td>2 x 10^{-4}</td>
<td>0.7 (0.0)</td>
</tr>
<tr>
<td>30</td>
<td>16.3 (16.1)</td>
<td>1 x 10^{-4}</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>3</td>
<td>0.6 (0.0)</td>
<td>1 x 10^{-5}</td>
<td>0.5 (0.0)</td>
</tr>
</tbody>
</table>

Concentrations of ferritin (µg/ml) and iron complexes (M) are tabulated at equivalent iron content, e.g., 1490 µg of ferritin per ml contain 280 µg of iron per ml (18.8% of ferritin) which is 5 x 10^{-3} M of an iron complex.

- Cells were treated with ferrous sulfate without chelate. Aquous stock of FeSO₄ was diluted directly in MEM.
- The glycine:iron molar ratio = 10; chromosome aberrations were not induced by glycine alone (5 x 10^{-3} to 10^{-4} M).
- The NTA:iron molar ratio = 2; chromosome data for cells treated with NTA alone are shown in Table 5.
- The histidine:iron molar ratio = 10; chromosome data for cells treated with histidine alone are shown in Table 6.
- The EDTA:iron molar ratio = 2; chromosome data for cells treated with EDTA alone are shown in Table 5.
- The cysteine:iron molar ratio = 10; cysteine alone at 5 to 10 x 10^{-3} M caused mitotic inhibition; chromosome data for cells treated with cysteine alone are shown in Table 3.
- No detectable mitotic and variable cell loss.
- Percentage of metaphase cells with at least one chromosome aberration; 50 to 300 metaphase cells were analyzed for chromosome- or chromatid-type exchanges and breaks. Whenever possible, at least 100 metaphase cells were scored.
- Numbers in parentheses, frequency (percentages) of chromosome- or chromatid-type exchanges per metaphase cell.
- MI, mitotic inhibition, less than one metaphase cell per 5000 cells; CL, cell loss with mitotic rate in the normal range, less than 50 metaphase cells countable.
- Controls, CHO cells exposed to MEM only, not treated with chemicals.
ferrous sulfate at $5 \times 10^{-3}$ M. Both breaks and exchanges (up to 20%) were observed in cells exposed to ferrous:NTA complexes at 3 and $2 \times 10^{-3}$ M, while a very low frequency of aberrations (1%) was seen in cells treated with ferric:NTA complexes. EDTA complexes of both Fe(II) and Fe(III) were cytotoxic and cytostatic at 5 and $3 \times 10^{-3}$ M. Chromosome aberrations (exchanges and breaks) were detected in cells exposed to $2 \times 10^{-3}$ M Fe(II):EDTA or Fe(III):EDTA complexes (Fig. 3). Ferrous:EDTA induced a higher frequency of aberrations than did ferric:EDTA (12.1 versus 2.7%). EDTA alone at concentrations above $10^{-2}$ M was found to cause cell loss without cytostatic activity.

Among the ligands examined, cysteine has dual properties as both a complexing and a reducing agent. Both chromosome breaks and exchanges were detected in cells treated with cysteine complexes of ferrous or ferric iron. The autooxidation of cysteine yielded insoluble cystine crystals which were detected in samples where cysteine concentrations exceeded 2 $\times 10^{-3}$ M.

During the 3-hr incubation period, cysteine caused some iron complexes. Histidine is a weak ligand for ferric iron, and precipitation of a ferric:histidine complex was observed over a wide range of iron concentrations ($10^{-4}$ to $5 \times 10^{-3}$ M). As well, complexes of iron with NTA and glycine formed precipitates at iron concentrations higher than $10^{-3}$ M.

Among the complexing agents which have higher binding constants for Fe(III), such as EDTA and NTA, higher levels of chromosomal damage (breaks and/or exchanges) were detected in the ferrous complexes than in the ferric ones. Conversely, histidine, which preferentially binds to Fe(II), was more cytotoxic as a ferric complex than as a ferrous complex.

Results in Tables 1 and 2 showed that neither the protein shell of ferritin nor iron complexes caused chromosome damage at comparable protein and iron concentrations to those in ferritin.

**Effect of Sulfhydryl Compounds, Glutathione and Cysteine.** Both cysteine and glutathione were effective in reducing the chromosome-damaging capacity of ferritin (Table 3). At $10^{-3}$ M, either sulfhydryl compound completely abolished the cytotoxic as well as the cytostatic action of high concentrations of ferritin (333 to 3330 µg/ml). In addition, glutathione and cysteine effectively reduced the frequencies of chromosome aberrations induced by lower concentrations of ferritin (33 to 167 µg/ml) to those in the nontreated cells.

A lower concentration of glutathione ($10^{-3}$ M) provided substantial protection from the clastogenic and cytotoxic activities of ferritin. In the presence of $10^{-3}$ M glutathione, the frequencies of chromosome aberrations induced by ferritin at 33 to 167 µg/ml were reduced to background levels. The cytostatic action of ferritin at 333 µg/ml was inhibited. In addition, $10^{-3}$ M glutathione partially protected cells treated with high concentrations of ferritin (667 to 3330 µg/ml); the cytotoxic effect of ferritin alone was lessened to mitotic inhibition.

**Effect of an Enediol Reducing Agent, Ascorbate.** In contrast to the effects of sulfhydryl reducing agents, ascorbate did not reduce the chromosome-damaging activity of ferritin (Table 4). Addition of ascorbate at $10^{-3}$ or $10^{-4}$ M with ferritin to cells did not alter the cytotoxic, cytostatic, or the clastogenic activities of ferritin. Cells exposed to a higher concentration of ascorbate ($10^{-2}$ M) alone or to a mixture of ferritin (16 to 360 µg/ml) and $10^{-2}$ M ascorbate showed mitotic inhibition. However, cells treated with a low dose of ferritin (4 µg/ml) and $10^{-2}$ M ascorbate exhibited normal mitotic rate and a background level of chromosome aberrations. This effect is similar to the reduced DNA damage observed in cells treated with $10^{-5}$ M iron:EDTA (equivalent to 3 µg of ferritin per ml) and $10^{-2}$ M ascorbate (28).

**Effect of Nitrogen and Oxygen Ligands, EDTA, NTA, and Salicylate.** Three chelating agents (EDTA, NTA, and salicylate) were examined for their effect on the induction of chromosome aberrations by ferritin (Table 5). EDTA was most effective in reducing the DNA-damaging action of ferritin. In the presence of $10^{-3}$ M EDTA (Table 5, Column D), the clastogenic, cytostatic, and cytotoxic effects of ferritin (15 to 750 µg/ml) were essentially cancelled. In comparison, $10^{-3}$ M NTA (Table 5, Column C) was less effective. It abolished the clastogenic and the cytotoxic effect of ferritin at 15 to 188 µg/ml. However, higher concentrations of ferritin (375 to 1880 µg/ml) remained cytotoxic. Salicylate at $10^{-3}$ M did not reduce either the cytotoxic or the clastogenic activity of ferritin (Table 5, Column B).

An increase in the concentration of chelating agents to $10^{-2}$ M produced a variety of responses. EDTA itself caused a loss of cells from the coverslips. NTA at $10^{-2}$ M (Table 5, Column F) showed a protective activity comparable to that of $10^{-3}$ M EDTA; the cytotoxic, cytostatic, and clastogenic effects of...
Ferritin (15 to 1880 \(\mu\)g/ml) were essentially abolished. Finally, 10^{-2} m salicylate (Table 5, Column E) did not inhibit the cytotoxic or clastogenic effects of ferritin. Salicylate may even have a slight enhancing effect on the chromosome-damaging action of ferritin at 15 and 30 \(\mu\)g/ml.

**Effect of Heterocyclic Nitrogen Ligands, Histidine and 2,2'-Bipyridyl.** The 2 nitrogen ligands histidine and bipyridyl affected the chromosome-damaging action of ferritin differently (Table 6). Histidine at 10^{-3} m or 10^{-2} m (Table 6) did not appreciably change the cytotoxic and cytoclastic effect of ferritin. Little alteration of the clastogenic activity of ferritin was detected with 10^{-3} m histidine. On the other hand, 10^{-2} m histidine reduced the chromosome aberrations induced by ferritin (35 \(\mu\)g/ml) from 13% to control levels.

In contrast, 2,2'-bipyridyl, which has a higher binding constant for ferrous iron than does histidine, increased the DNA-damaging action of ferritin (Table 6). For example, addition of 10^{-3} m bipyridyl with ferritin (19 \(\mu\)g/ml) to cells resulted in an increase of damage from 10.7% aberrations to mitotic inhibition. Ferritin at low concentration (5 \(\mu\)g/ml) was not clastogenic, and addition of bipyridyl caused an increase in the aberrations to 33.5% with 50% chromosome- and chromatid-type exchanges. 2,2'-Bipyridyl alone at 10^{-2} m also induced low levels of chromosome aberrations (6%) which were reduced by low concentrations of ferritin (1 and 2 \(\mu\)g/ml).

**Effect of Catalase and Superoxide Dismutase.** Catalase and superoxide dismutase had little or no effect on the frequency of chromosome aberrations induced by ferritin (Table 7). Neither catalase (100 \(\mu\)g/ml) nor superoxide dismutase (100 \(\mu\)g/ml) detectably reduced (a) the toxic or cytoclastic effects of high concentrations of ferritin (333 to 1670 \(\mu\)g/ml) or (b) the frequency of aberrations caused by ferritin at 167 and 67 \(\mu\)g/ml. There may be a marginal protective effect of catalase and superoxide dismutase in cells treated with ferritin at 33 \(\mu\)g/ml.

**DISCUSSION.**

The data presented in this report demonstrate that exposure of mammalian cells to ferritin caused severe chromosomal damage. This raises questions of (a) the nature of the active component in ferritin which causes the damage and (b) the mechanism of the reaction which leads to chromosome abnormalities. As a partial answer to the first question, we found that neither apoferritin (Table 1) nor complexes of iron [as Fe(II) or Fe(III) (Table 2)] individually could account for the high cytotoxic and clastogenic activity of ferritin. Thus, the intact ferritin molecule appears to be required for DNA damage.

To examine the possible mechanism of mutagenic action of ferritin, we have determined the effect of chemicals which modify the physiological activities of ferritin. Chromosome damage could arise during mobilization of iron, during oxidation-reduction of ferritin iron, or by a catalytic action of ferritin.

If mobilization of iron is important in the mechanism of damage, then agents which affect mobilization should also affect chromosome damage. Mobilization of ferritin iron is enhanced by reducing agents. Physiological reducing agents

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**Table 5**

**Effects of EDTA, NTA, and salicylate on chromosome aberrations induced by ferritin**

<table>
<thead>
<tr>
<th>Ferritin concentration ((\mu)g/ml)</th>
<th>Ferritin alone (A)</th>
<th>Salicylate (B)</th>
<th>EDTA (C)</th>
<th>NTA (D)</th>
<th>Ferritin plus chelating agents (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1860 Toxic</td>
<td>Toxic</td>
<td>Toxic</td>
<td>2.1 (2.1)</td>
<td>0.5 (0.0)</td>
<td>Toxic</td>
</tr>
<tr>
<td>750 Toxic</td>
<td>Toxic</td>
<td>MI</td>
<td>19.4 (11.3)</td>
<td>0.6 (0.0)</td>
<td>MI</td>
</tr>
<tr>
<td>375 MI</td>
<td>1.4 (0.0)</td>
<td>0.0 (0.0)</td>
<td>32.6 (52.8)</td>
<td>0.6 (0.0)</td>
<td>19.6 (23.0)</td>
</tr>
<tr>
<td>15 12.1 (10.6)</td>
<td>0.9 (0.0)</td>
<td>0.6 (0.0)</td>
<td>12.9 (18.6)</td>
<td>0.7 (0.0)</td>
<td>0.8 (0.0)</td>
</tr>
<tr>
<td>3 3.2 (2.7)</td>
<td>0.9 (0.0)</td>
<td>0.5 (0.0)</td>
<td>0.8 (0.0)</td>
<td>0.6 (0.0)</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>0 0.6 (0.0)</td>
<td>0.8 (0.0)</td>
<td>0.6 (0.0)</td>
<td>0.5 (0.0)</td>
<td>0.5 (0.0)</td>
<td>0.5 (0.0)</td>
</tr>
</tbody>
</table>

**Table 6**

**Effect of histidine and 2,2'-bipyridyl on chromosome aberrations induced by ferritin**

<table>
<thead>
<tr>
<th>Ferritin concentration ((\mu)g/ml)</th>
<th>Ferritin alone (A)</th>
<th>Ferritin plus chelating agent (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>Toxic</td>
<td>Toxic</td>
</tr>
<tr>
<td>1500 Toxic</td>
<td>Toxic</td>
<td>Toxic</td>
</tr>
<tr>
<td>700 MI</td>
<td>MI</td>
<td>MI</td>
</tr>
<tr>
<td>350 52.9 (58.8)</td>
<td>60.8 (73.3)</td>
<td>61.5 (69.2)</td>
</tr>
<tr>
<td>150 38.7 (58.1)</td>
<td>47.5 (45.6)</td>
<td>33.8 (47.9)</td>
</tr>
<tr>
<td>35 13.0 (11.2)</td>
<td>15.6 (11.1)</td>
<td>0.9 (0.9)</td>
</tr>
<tr>
<td>15 2.9 (2.9)</td>
<td>0.7 (0.0)</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>3 0.6 (0.0)</td>
<td>0.7 (0.0)</td>
<td>0.8 (0.0)</td>
</tr>
<tr>
<td>0 0.5 (0.0)</td>
<td>0.6 (0.0)</td>
<td>0.5 (0.0)</td>
</tr>
<tr>
<td>2,2'-Bipyridyl</td>
<td>Toxic</td>
<td>44.5 (83.3)</td>
</tr>
<tr>
<td>96 47.2 (31.7)</td>
<td>Toxic</td>
<td>10.7 (7.1)</td>
</tr>
<tr>
<td>47</td>
<td>MI</td>
<td>2.4 (3.2)</td>
</tr>
<tr>
<td>19</td>
<td>66.7 (95.0)</td>
<td>33.5 (50.7)</td>
</tr>
<tr>
<td>10 0.5 (0.0)</td>
<td>3.5 (3.5)</td>
<td>1.6 (6.5)</td>
</tr>
<tr>
<td>1 0.5 (0.0)</td>
<td>6.2 (14.1)</td>
<td></td>
</tr>
</tbody>
</table>
The second aspect is the effect of chelating agents to catalyze oxidation-reduction of iron. EDTA and NTA enhance the oxidation of Fe(II), while bipyridyl can slowly reduce Fe(III) (3). DNA damage may be related to the amount of Fe(II) present in or near ferritin, and this would be decreased by EDTA and NTA and increased by bipyridyl.

A third relationship is that between the protective effect and the "denticity" of the chelating agents. EDTA, NTA, and salicylate have similar effective stability constants for Fe(III), log \( K_{\text{eff}} = 8.0 \) to 8.2 (17). Their ability to suppress the chromosome-damaging action of ferritin is correlated with the denticity (number of ligand-forming groups) of these compounds. EDTA (hexadentate) was the most effective inhibitor followed by the tetradentate NTA. Salicylate (bidentate) did not show inhibition. The protective effect may be related to the rate of ligand exchange. Further research is required to identify which of these factors (relative stability constants for Fe(II) and Fe(III), rate of oxidation-reduction, and denticity) are most important in affecting the mutagenicity of ferritin.

We observed strong inhibitory activities of glutathione and cysteine on DNA damage caused by ferritin (Table 3). Sulfhydryl compounds glutathione and cysteine are very reactive towards electrophilic species, including many free radicals (15). They are able to trap not only reactive hydroxyl radicals but also other less reactive radicals such as those found in damaged lipids, proteins, and DNA. The observed protective activity of the thiols appears to reflect more their radical-trapping activity than their reducing or chelating properties. Other reducing agents such as ascorbate, which lacks such strong trapping properties, did not exhibit similar inhibition (Table 4). In addition, other ligands such as histidine and 2,2'-bipyridyl (Table 6), which are like thiols in preferentially stabilizing iron in ferrous state, either showed weak protection or enhanced the clastogenic action of ferritin (Table 6).

The protective effect of cysteine against the clastogenic activity of ferritin is in contrast to the chromosome-damaging action of iron:cysteine mixtures (Table 2). Free iron catalyzes the oxidation of cysteine (13), and an oxidation product of the reaction may cause the DNA damage. Bound iron in the ferritin core does not appear to have this catalytic activity towards cysteine. Glutathione is different from cysteine in that free iron does not catalyze its oxidation (13), and iron:glutathione mixtures did not cause chromosome aberrations.\(^5\)

Ascorbate also interacts differently with ferritin than with free iron. Ascorbate had no significant effect on the clastogenic action of ferritin (Table 4). In contrast, ascorbate reacted with iron:EDTA complexes to cause severe chromosome aberrations (28).

Reduced oxygen species such as superoxide radicals (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)), and hydroxyl radicals (-OH) are formed during the oxidation-reduction of iron compounds (7, 8, 18) and may be responsible for the toxic and clastogenic actions of ferritin. To test the possible involvement of superoxide radicals and hydrogen peroxide, we examined the effect of superoxide dismutase and catalase. Neither superoxide dismutase nor catalase influenced the chromosome-damaging activity of ferritin (Table 7). This indicates that either there was no appreciable accumulation of these species or the species were not substantially involved in the mechanism of damage.

\(^5\) R. F. Whiting, L. Wei, and H. F. Stich, unpublished data.
Moreover, superoxide radicals and H$_2$O$_2$ were not detected during deposition of ferritin iron (3, 29). It is possible that low steady-state amounts of these or related species are present, particularly inside the ferritin shell which is inaccessible to enzymes. A possible mechanism of DNA damage by ferritin is the formation of hydroxyl radicals (·OH) during reduction and reoxidation of ferritin iron. These latter processes occur continuously at the catalytic sites (Reactions A and B) (3, 14, 29). It has been proposed (3) that ferric-peroxy species are formed during oxidation-deposition of iron in ferritin (Reaction C). These peroxo species could react with Fe(II) or other reductants to produce hydroxyl radicals in a Fenton-type reaction (Reaction D) (7, 8, 18).

$$\text{Fe(III)}_{\text{bound}} + \text{reductant} \rightleftharpoons \text{Fe(II)}_{\text{bound}} + \text{oxidant} \quad \text{(A)}$$

$$\text{Fe(II)}_{\text{bound}} \rightleftharpoons \text{Fe(II)}_{\text{free}} \quad \text{(B)}$$

$$\text{Fe(II)}_{\text{bound}} + \text{O}_2 \rightleftharpoons \text{Fe(II)} \cdot \cdot \cdot \text{O}_2 \cdot \cdot \cdot \text{Fe(II)} \rightleftharpoons \text{Fe(III)} - \text{O} - \text{O} - \text{Fe(III)} \quad \text{Fe(III) - O - O} - \text{Fe(III)} + \text{Fe(II)} + 2\text{H}_2\text{O} \rightleftharpoons 3\text{Fe(II)-OH} + \cdot \text{OH} \quad \text{(C)}$$

$$\text{(D)}$$

However, ·OH is very short lived, and free ·OH would be scavenged by salicylate [salicylate had no protective effect (Table 5)]. Thus, only secondary radicals or other reactive species may escape from the ferritin molecule to react with DNA. It may be these species which are trapped by glutathione and cysteine.

Chemical studies have demonstrated that mobilization of ferritin iron by dihydroriboflavin nucleotides (dihydroriboflavin mononucleotide and dihydroriboflavin adenine dinucleotide) is rapid and quantitative (4, 14, 25). Our preliminary results of the NADH:FMN system in CHO cells showed that, at millimolar levels, NADH:FMN was cytotoxic (10$^{-3}$ M NADH:10$^{-3}$ M FMN), cytostatic (10$^{-3}$ M NADH:10$^{-4}$ M FMN), and clastogenic (10$^{-4}$ M NADH:10$^{-3}$ M FMN) to CHO cells (Table 8). No chromosome damage was caused by lower concentrations of the reduced flavins (10$^{-3}$ M NADH:10$^{-4}$ M FMN), by NADH alone (10$^{-4}$ to 10$^{-2}$ M), or FMN alone (10$^{-4}$ to 10$^{-3}$ M) (Table 8). However, addition of any of these latter agents (10$^{-4}$ M NADH:FMN, NADH, or FMN alone) to ferritin did not alter the frequency of chromosome aberrations induced by ferritin (data not shown). NADH:FMN is a hydroxylating system (25). This hydroxylating property of the reduced flavins makes it difficult to determine the role of dihydroribavin mononucleotide in the mechanism of damage by ferritin.

Finally, our results indicate that ferritin concentrations in normal human serum (0.01 to 0.1 µg/ml) and tissues (up to 5 µg/ml) are below the levels causing chromosome damage in cells (6, 9, 21). However, in cancer and other pathological states, serum ferritin concentration can reach 12 µg/ml (21, 23), which is near the lower limit of clastogenic concentration of ferritin. In addition, an in vitro study showed that ferritin concentration as low as 0.25 µg/ml caused a marked suppression of phytohemagglutinin and concanavalin A-induced blastogenesis in human lymphocytes (19). Furthermore, kinetic studies of ferritin mobilization show that the rate of iron release is dependent on the age and the fullness of the iron core. Newly deposited iron is released faster (9). The maximum rate was found in ferritin molecules which were less than one-third full (1200 iron atoms per ferritin molecule) (5, 14). This may imply that ferritins from different sources or with different iron contents would have varied clastogenic activity. Moreover, normal physiological levels of glutathione (10$^{-4}$ to 5 x 10$^{-3}$ M) (15) are likely to be sufficient in suppressing any cytotoxic or clastogenic activity of ferritin.

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REFERENCES


Chromosome-damaging Activity of Ferritin and Iron Compounds

Fig. 1. Metaphase plate of a CHO cell exposed to MEM only, not treated with chemicals.

Fig. 2. Metaphase plate of a CHO cell exposed to ferritin (30 μg/ml) for 3 hr and harvested 20 hr posttreatment, showing one chromatid-type interchange.

Fig. 3. Metaphase plate of a CHO cell exposed to Fe(II):EDTA (2 x 10^-3 M), showing chromatid-type interchanges and breaks.

Fig. 4. Metaphase plate of a CHO cell exposed to ferritin (60 μg/ml), showing multiple exchanges and breaks.
Chromosome-damaging Activity of Ferritin and Its Relation to Chelation and Reduction of Iron

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