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Cellular Pool of Transient Ferric Iron, Chelatable by Deferoxamine and Distinct from Ferritin, that Is Involved in Oxidative Cell Injury

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SUMMARY

A cellular pool of transient ferric iron that is chelatable by deferoxamine, distinct from ferritin, and required for oxidative cell injury has been identified in cultured rat hepatocytes labeled with $^{59}FeCl_3$. Pretreatment of hepatocytes with deferoxamine depleted the cellular pool of chelatable iron and protected the cells from an oxidative injury. Incubation of deferoxamine-pretreated hepatocytes in serum-free medium restored both the chelatable iron pool and the susceptibility to oxidative injury. Furthermore, inhibition of protein degradation with chymostatin prevented the restoration of both the chelatable pool and susceptibility to oxidative injury. The deferoxamine-chelatable iron pool was distinguished kinetically and immunochemically from the larger cellular pool of ferritin iron. The labeled iron in the deferoxamine-chelatable pool was transient, unlike either the total cellular uptake of ^{59}Fe or its incorporation into ferritin, both of which increased with time of labeling. With pulse-chase labeling, the percentage of the total uptake of ^{59}Fe that was represented by the deferoxamine-chelatable pool decreased. At the same time, the percentage represented by radioactivity immunoprecipitable as ferritin increased. Furthermore, immunoprecipitation of ferritin from the labeled lysates enriched the resulting immunosupernatants in deferoxamine-chelatable iron. The degree of enrichment for chelatable iron correlated with the percentage of the cellular label that was immunoprecipitable as ferritin. The deferoxamine-chelatable iron appears to represent a metabolically common pool of iron that is rapidly in transit through the cell. Extracellular iron entering the pool can be utilized for heme synthesis or stored in ferritin, whereas protein degradation releases storage iron into this pool.

The killing of cultured rat hepatocytes by either hydrogen peroxide or TBHP depends on a cellular source of both ferric iron and superoxide anions (1–5). Presumably, superoxide anions reduce a cellular pool of ferric iron to ferrous iron. In turn, ferrous iron reacts with either hydrogen peroxide or TBHP to form a more potent oxidizing species. Thus, as a chelator of ferric iron, DF can prevent the cell killing by either peroxide. Importantly, the hepatocytes can be pretreated with DF, washed thoroughly, and then exposed to the peroxide. This result implies that DF chelates a cellular pool of iron, rather than extracellular iron present in the medium.

The likely source of this pool is the iron that enters and leaves ferritin, the major iron storage protein in virtually all cells. Two mechanisms are proposed for the mobilization of iron from ferritin. The first is the long held belief that the release of iron from ferritin occurs by the reduction of ferric to ferrous iron, without a requirement for the degradation of the apoprotein. Under cell-free conditions, some potentially physiologic, and many clearly nonphysiologic, reducing agents release iron from ferritin. It has been proposed that an oxidative stress itself may reductively release iron from ferritin, thereby promoting cell injury (6–8). In particular, superoxide anions, generated by xanthine oxidase or by stimulated polymorphonuclear leukocytes, reductively released iron from ferritin (6, 7). Furthermore, the redox cycling of paraquat promoted iron release from ferritin, an effect attributed to both the generation of superoxide anions and the paraquat free radical itself (7, 8).

The second mechanism by which iron is liberated from ferritin is a consequence of the degradation of this protein. Incubation of DF-pretreated hepatocytes in a serum-free medium containing only 0.25 nM iron restored the sensitivity of the cells to TBHP within 4–6 hr (4). Furthermore, conditions that perturbed the autophagic degradation of ferritin affected the regeneration of the iron pool required for oxidative cell killing. These conditions included both the activation of auto-

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ABBREVIATIONS: TBHP, *tert*-butyl hydroperoxide; BSA, bovine serum albumin; DF, deferoxamine; HBB, HEPES-borate buffer (0.25 M boric acid, 10 mM HEPES, pH 7.3, 0.5% Triton X-100); HBS, HEPES-buffered saline (0.15 M NaCl, 10 mM HEPES, pH 7.3); BPS, bathophenanthrolene disulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

phagic protein degradation by amino acid starvation and its inhibition by a variety of pharmacologic manipulations (4).

Thus, the autophagic degradation of ferritin most likely contributes to the physiologic origin of the iron pool required for oxidative cell injury. More broadly, DF-chelatable iron may represent a pool through which cellular ferric iron passes during its uptake, storage, and mobilization. In the present paper an assay was developed to quantitate the DF-chelatable ferric iron pool. The data presented here document that this pool of transient ferric iron is distinct from ferritin and is required for the oxidative injury of hepatocytes.

Experimental Procedures

Materials. Affigel 10, Chelex 100 (100/200, K^+), and Immunobead reagent (goat anti-rabbit immunoglobulin) were obtained from Bio-Rad (Richmond, CA). Dexamethasone, chymostatin, 3-methylcholanthrene, TBHP, BSA (fraction V, protease free), and the reagents for the bicinchoninic acid protein assay were purchased from Sigma (St. Louis, MO). Anhydrous methanol (>99%) was from Aldrich (Milwaukee, WI), $^{59}FeCl_3$ (1 mCi/ml of 0.1 M HCl) from Amersham (Arlington Heights, IL), and desferal (DF mesylate, USP) from Ciba Pharmaceuticals (Summit, NJ). Williams' E medium was obtained from GIBCO Laboratories (Chagrin Falls, OH), collagenase from Boehringer Mannheim (Indianapolis, IN), and rabbit anti-horse ferritin immunoglobulin and normal rabbit immunoglobulin from Dako (Carpinteria, CA). The ability of the rabbit anti-horse ferritin immunoglobulin to immunoprecipitate rat ferritin was shown previously (4).

Preparation and culture of hepatocytes. Male Sprague-Dawley rats (150–200 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA). On the day before preparation of hepatocytes (except where indicated in the text), rats were injected intraperitoneally with 3-methylcholanthrene (25 mg/kg of body weight), as a 10 mg/ml solution in corn oil. For one experiment, sodium phenobarbital (80 mg/kg of body weight/day) instead was administered for 3 days before cell preparation. All animals were fed *ad libitum* and fasted overnight before hepatocyte isolation. Hepatocytes were prepared by collagenase perfusion (9). Yields of 3–5 × 10^8 cells/liver (85–90% viability by trypan blue exclusion) were routinely obtained. The hepatocytes (2 × 10^6) were plated in 25-cm^2 polystyrene tissue culture flasks, in a volume of 3 ml of Williams' E medium supplemented with 10% heat-inactivated (55°, 30 min) fetal bovine serum, 1 μg/ml dexamethasone, 0.02 units/ml insulin, 10 IU/ml penicillin, 10 μg/ml streptomycin, and 50 μg/ml gentamycin (complete Williams' E medium). After a 2-hr incubation at 37° in an atmosphere of 5% CO_2/95% air, the flasks were washed twice with a prewarmed isotonic HEPES-buffered solution (142 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, 10 mM HEPES, pH 7.2) to remove nonadherent cells. Incubation continued overnight with the remaining adherent cells in 5 ml of complete Williams' E medium.

Labeling with $^{59}FeCl_3$. A ^{59}Fe-labeling solution was prepared by diluting the stock solution of $^{59}FeCl_3$ (in 0.1 M HCl) with 2 volumes of serum-supplemented Williams' E medium, to a final concentration of 100 μCi/ml. After approximately 30 min at an acidic pH, the labeling solution was neutralized by further dilution with 20 volumes of complete Williams' E medium. Most of the ^{59}Fe label was recovered as transferrin after the addition of $^{59}FeCl_3$ to serum (10).

Hepatocytes in 3 ml of complete Williams' E medium were incubated with 15 μCi of the ^{59}Fe-labeling solution. After labeling, hepatocytes were washed three times with isotonic HEPES-buffered solution, and incubation continued in fresh complete Williams' E medium, but without serum, as described in the figure legends. Alternatively, 1 ml of 0.5% Triton X-100 in HBS was added to each flask of washed cells, which were then lysed overnight at 4°. Typically, cultures were analyzed in triplicate.

Preparation of Affigel-DF. Affigel 10 was washed extensively with anhydrous methanol and then suspended in an equal volume of methanol containing a 75% molar excess of DF. After gentle agitation for 4 hr at room temperature, the methanolic supernatant containing unreacted DF was separated from the gel, which was then quenched with an equal volume of 0.1 M ethanolamine in methanol. After an additional 1 hr, the gel was washed extensively with water. Finally, the settled gel was diluted with 2 volumes of water.

The amount of DF bound to the gel was calculated from the amount of unreacted soluble DF remaining in the initial methanolic supernatant. The amount of soluble DF was determined colorimetrically at 444 nm after reaction of the methanolic supernatant with 2 mM $FeCl_3$/250 mM citric acid (approximately 5 min at room temperature). Typically, this procedure resulted in binding of 12 μmol of DF/ml of settled gel.

Chromatographic analysis of DF-chelatable ^{59}Fe. Aliquots (2 ml) of the suspension of Affigel-DF (1:3 dilution of the settled gel) were packed in miniature polypropylene columns (Poly-Prep columns; Bio-Rad). Before use, the packed columns were treated with 6 ml of equilibration buffer (2% BSA in HBB) to reduce nonspecific binding.

A 1/4 volume of sample buffer (2.5% BSA, 0.25% phenol red, 1.25 M boric acid, 50 mM HEPES, pH 7.3, 0.5% Triton X-100) was added to the ^{59}Fe-labeled Triton lysates, for a total volume of 200 μl. The buffered sample was slowly adsorbed onto Affigel-DF. After 1 hr at room temperature, unbound ^{59}Fe was washed off the column with 12–24 ml of wash buffer (0.5% BSA in HBB), collected in fractions of 6 ml. Typically, lysates from cultures labeled overnight required more extensive washing of the Affigel-DF columns. To elute the bound ^{59}Fe from the column, a solution of sodium dithionite was freshly prepared and used within 2 min. First, to chelate contaminating iron, crystalline dithionite (final concentration of 200 mM) was vortexed briefly with 5 mM EDTA in HBS. The resulting solution then was diluted with an equal volume of 2 mM BPS in HBS. A 3-ml aliquot of this final solution was added to each column and, after 10 min, the remainder of the red-colored Fe(II)-BPS complex was eluted from the column with an additional 9–15 ml of HBS.

The results were calculated as cpm of ^{59}Fe bound/mg of protein applied to the column. Unless otherwise noted, results were reported as specific binding, determined by subtracting cpm bound nonspecifically. Nonspecific binding was determined by analyzing a duplicate aliquot of the lysate that additionally had been pretreated with 100 mM DF for 1 hr at 4° before chromatographic analysis for chelatable ^{59}Fe. Recoveries were routinely >90% of the ^{59}Fe applied. Consequently, the percentage of cellular ^{59}Fe binding to the column could be calculated more conveniently from the ratio of bound cpm to total recovered cpm. All ^{59}Fe-labeled samples were counted in an LKB 1282 Compugamma CS counter equipped with a 3-inch crystal.

Immunoprecipitation. Ferritin was immunoprecipitated in a buffer containing 0.2% BSA in HBS and 0.5% Triton X-100. The ^{59}Fe-labeled Triton lysate was incubated first with a saturating titer of antiferritin immunoglobulin. To correct for nonspecific immunoprecipitation, an additional aliquot of the lysate was incubated with normal immunoglobulin at a protein concentration equivalent to that of the antiferritin immunoglobulin used for immunoprecipitation. After 1 hr at 4°, a saturating amount of Immunobead reagent was added, and the mixture was agitated gently overnight at 4°. The immunoprecipitate was then centrifuged and washed three times with 0.2% BSA in HBS/0.5% Triton X-100.

Other assays. Protein was assayed by the bicinchoninic acid method (11), using BSA as a protein standard. The extent of cell injury from exposure to TBHP was assessed from the activity of cellular lactate dehydrogenase released into the culture medium (12).

Results

Assay for the DF-chelatable pool of ferric iron. After its covalent linkage to agarose (Affigel-DF), DF retained its high specificity for ferric iron. This was shown by the experiment illustrated in Fig. 1. A Triton X-100 lysate, prepared from

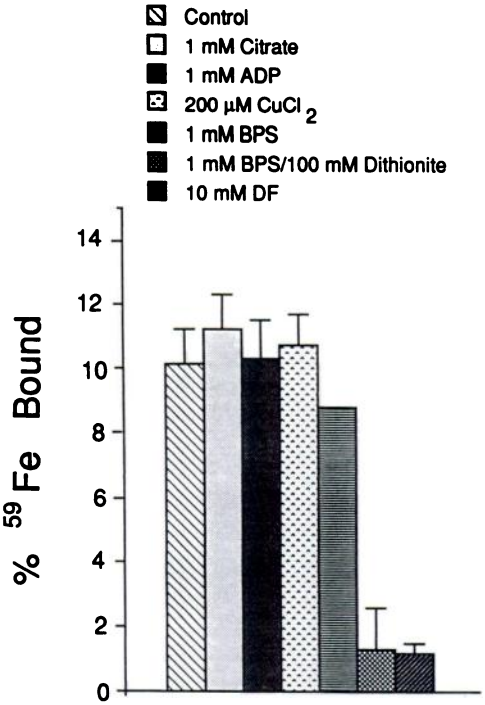

Fig. 1. Affigel-DF specifically binds a cellular pool of ferric iron. Cultured hepatocytes were labeled with $^{59}FeCl_3$ for 30 min and then lysed with Triton X-100. Aliquots of the lysate were supplemented as indicated in the figure and then applied to columns of Affigel-DF. Dithionite was syringe-filtered through 2 ml of Chelex before addition of BPS. The respective samples were assayed in duplicate.

cultured rat hepatocytes labeled with $^{59}FeCl_3$ for 30 min, was passed over a column of Affigel-DF. Approximately 10% of the applied radioactivity was retained by the column. Treatment of the lysate with soluble DF before its chromatography on Affigel-DF decreased the retention of radioactivity by >90%.

In contrast, treatment of the same lysate with the ferrous iron chelator BPS did not affect the retention of radioactivity by the column. However, when the lysate was treated with BPS together with dithionite, a reductant that converts ferric to ferrous iron, the radioactivity retained by the column was reduced by 90%. The radioactivity bound to the column after treatment of the lysate with dithionite plus BPS was equivalent to that retained after treatment of the lysate with soluble DF and presumably represents nonspecific binding of the cellular label. Thus, almost all of the radioactivity in the lysate that bound specifically to Affigel-DF represented iron in the ferric state.

The addition of weak iron chelators, citrate or ADP, to the lysates did not reduce the retention of ^{59}Fe by Affigel-DF (Fig. 1). In addition, the binding of cellular ^{59}Fe was not inhibited by an excess of cupric ions, another redox-active transition metal (Fig. 1). The inability of copper to influence the binding of ferric iron to Affigel-DF is consistent with the fact that the association constants of these transition metals for DF are 10^{31} and 10^{14} for iron and copper, respectively (13).

Kinetics of the labeling of the iron pool measured by Affigel-DF chromatography. Fig. 2 compares the time course of the labeling of the pool of ^{59}Fe that binds to Affigel-DF with both the total cellular uptake of radioactivity and the ^{59}Fe incorporated into ferritin. After a 15-min incubation of the cultured hepatocytes with $^{59}FeCl_3$, 10% of the radioactivity in a Triton X-100 lysate bound to the Affigel-DF column. Similarly, after 15 min of labeling, the incorporation of ^{59}Fe into immunoprecipitable ferritin also was 10% of the total uptake. However, as the hepatocytes were labeled for progressively longer time periods, a decreasing percentage of the total ^{59}Fe in the cell lysate was retained by the Affigel-DF column. At the same time, an increasing percentage of the total radioactivity was immunoprecipitable as ferritin.

After incubation of the cells with ^{59}Fe for 3 hr, approximately 2% of the total radioactivity in the lysate bound to Affigel-DF, whereas >70% of the total represented ^{59}Fe incorporated into ferritin. Steady state was obtained after 3 hr, inasmuch as continued incubation for 24 hr produced a substantial increase in the total radioactivity accumulated by the cells, without significant changes in the percentages of this total represented by ^{59}Fe that was either chelatable by DF or immunoprecipitable as ferritin.

The results of a pulse-chase experiment shown in Fig. 3 further document the rapid turnover of the DF-chelatable pool. The hepatocytes were labeled with ^{59}Fe for 15 min, washed, and placed in fresh medium. At this time, about 9% of the total radioactivity in a cell lysate bound to Affigel-DF. After a 10-min chase, this value fell to <5% (Fig. 3A), whereas the radioactivity immunoprecipitable as ferritin increased by >10% (Fig. 3B).

The addition of DF to the culture medium during the 10-min chase rapidly depleted the cultures of chelatable ^{59}Fe (Fig. 3A) and prevented the incorporation of radioactivity into ferritin (Fig. 3B). In the presence of DF, there was a loss of 4% of the ^{59}Fe that was immunoprecipitable as ferritin. This result is consistent with a report that only the labeled iron most recently incorporated into ferritin may be accessible to DF (14).

The labeling kinetics of the ^{59}Fe pool retained by Affigel-DF varied with the metabolic demand for iron. Treatment of rats with either phenobarbital or 3-methylcholanthrene increases the hepatic content of cytochrome P-450. Fig. 4 shows that the pulse labeling of hepatocytes prepared from rats treated with either phenobarbital or 3-methylcholanthrene tripled the percentage of the total cellular ^{59}Fe that was bound by Affigel-DF.

Evidence that the iron pool chelatable by DF is distinct from ferritin. Hepatocytes were labeled with ^{59}Fe either overnight, to steady state (Fig. 5), or with a 20-min pulse (Fig. 6). The cells were then lysed with Triton X-100, and the lysates were treated with antiferritin immunoglobulin. The immunosupernatant was quantitatively depleted of ferritin, because no further radiolabel was immunoprecipitated upon treatment of the immunosupernatant with additional antiferritin immunoglobulin (data not shown). As a control, the same lysate was treated with a normal immunoglobulin (no known antigenic specificity), which nonspecifically immunoprecipitated 1–2% of the total cellular label (Figs. 5A and 6A).

Sixty percent of the intracellular ^{59}Fe was immunoprecipitable as ferritin upon pulse labeling (Fig. 6A). This value increased to 90% at steady state (Fig. 5A). For comparison, 15% and 2% of the intracellular ^{59}Fe was chelatable by DF upon pulse labeling or steady state labeling, respectively. These values are similar to those presented in Fig. 2.

Figs. 5B and 6B indicate that the immunosupernatants, obtained by treatment of the lysates with either the antiferritin antibody or the normal immunoglobulin, contained similar quantities of ^{59}Fe that were chelatable by DF. In other words,

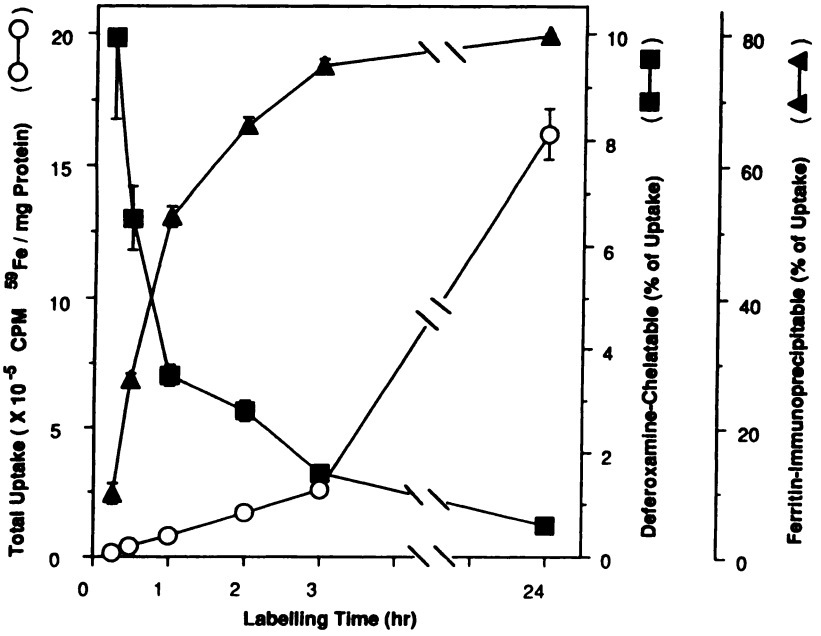

Fig. 2. Kinetics of the labeling of cellular iron pools. After incubation with $^{59}FeCl_3$ for the times indicated, cultured hepatocytes were lysed with Triton X-100, and the total uptake of cellular ^{59}Fe was determined by counting an aliquot of the lysate. The intracellular distribution of ^{59}Fe was assayed for the percentage that was chelatable by DF, as well as the percentage of label that was immunoprecipitable as ferritin.

the antiferritin antibody did not remove any more DF-chelatable iron than did the control incubation with normal immunoglobulin. This result was obtained despite the fact that the antiferritin antibody quantitatively depleted the immunosupernatants of ferritin.

However, with respect to the original lysate, the antiferritin antibody enriched the immunosupernatants in ^{59}Fe retained by Affigel-DF (compare Fig. 5, B and C). This enrichment of the immunosupernatant was a consequence of depletion of ferritin, which represents 90% of the steady state label, from the lysates. Accordingly, a lesser enrichment in chelatable iron was observed in the immunosupernatants after pulse labeling of the hepatocytes (compare Fig. 6, B and C). With pulse labeling, ferritin accounted for 60% of the total cellular ^{59}Fe (Fig. 6A). Importantly, the control incubation with normal immunoglobulin did not enrich immunosupernatants from steady state or pulse labeled cells in ^{59}Fe retained by Affigel-DF (compare Fig. 5, B and C and Fig. 6, B and C).

Ferric iron stored in ferritin was essentially not chelatable by DF. This is demonstrated by the fact that the control incubation with normal immunoglobulin immunoprecipitated a similar quantity of chelatable ^{59}Fe from the Triton lysate as did the antiferritin antibody (Figs. 5B and 6B). This result was obtained despite the fact that the antiferritin antibody immunoprecipitated 90% of the ^{59}Fe in the lysate after steady state labeling of the cells (Fig. 5A). In contrast, the control incubation with normal (nonspecific) immunoglobulin immunoprecipitated only 1–2% of the total label from the same lysates. Thus, the radioactivity chelatable by DF from the immunoprecipitates (Figs. 5B and 6B) presumably is not iron stored in ferritin.

Interestingly, the small fraction of the total cellular ^{59}Fe immunoprecipitated in the control incubation with normal immunoglobulin (Figs. 5A and 6A) was enriched in DF-chelatable iron (compare Fig. 5, B and C and Fig. 6, B and C). In other words, the control incubation with normal immunoglobulin preferentially immunoprecipitated the cellular pool of DF-chelatable iron. As emphasized above, ferritin represented a greater percentage of the ^{59}Fe in the lysates after steady state labeling, as opposed to pulse labeling. Accordingly, the immunoprecipitates from lysates of steady state labeled hepatocytes obtained from the control incubation with normal immunoglobulin were more greatly enriched in chelatable iron than were similar immunoprecipitates from pulse labeled cells (compare Fig. 5, B and C and Fig. 6, B and C). Inspection of Figs. 5C and 6C reveals that the immunoglobulin that enriches the immunoprecipitates (normal immunoglobulin) differs in specificity from the immunoglobulin that enriches the immunosupernatants (antiferritin immunoglobulin).

Association of the DF-chelatable iron pool with oxidative cell injury. Treatment of cultured hepatocytes with DF prevents cell killing by TBHP (3–5). Sensitivity to TBHP is restored by incubation of the cells in the virtual absence of an exogenous source of iron (0.25 nM iron) (4). Fig. 7A shows that the cellular pool of chelatable ferric iron depleted by treatment with DF was similarly restored by incubation of the hepatocytes in the absence of exogenous iron.

Cultured hepatocytes were labeled with ^{59}Fe for 18 hr, washed, and treated with DF for 1 hr. Control cultures were not treated with DF. From a cell lysate, the radioactivity retained by Affigel-DF was reduced by 70% after treatment of the cells with DF (Fig. 7A). Fig. 7B shows that hepatocytes were resistant to the toxicity of TBHP after a similar pretreatment with DF.

Incubation of the DF-treated cells in serum-free medium for 4 hr before preparation of the lysates restored the radioactivity retained on an Affigel-DF column to 140% of the untreated control value (Fig. 7A). After the same 4-hr incubation, 50% of the sensitivity to TBHP was restored (Fig. 7B). Although the chelatable iron pool was restored to a greater extent than was the sensitivity to TBHP (Fig. 7, A versus B), this difference may reflect a difference in the labeling of this pool rather than its true size. The DF-chelatable iron pool may be labeled to a higher specific activity during its regeneration from ferritin than by the uptake of exogenous ^{59}Fe.

Inhibitors of autophagic protein degradation, such as chymostatin, inhibit the restoration of sensitivity to TBHP after

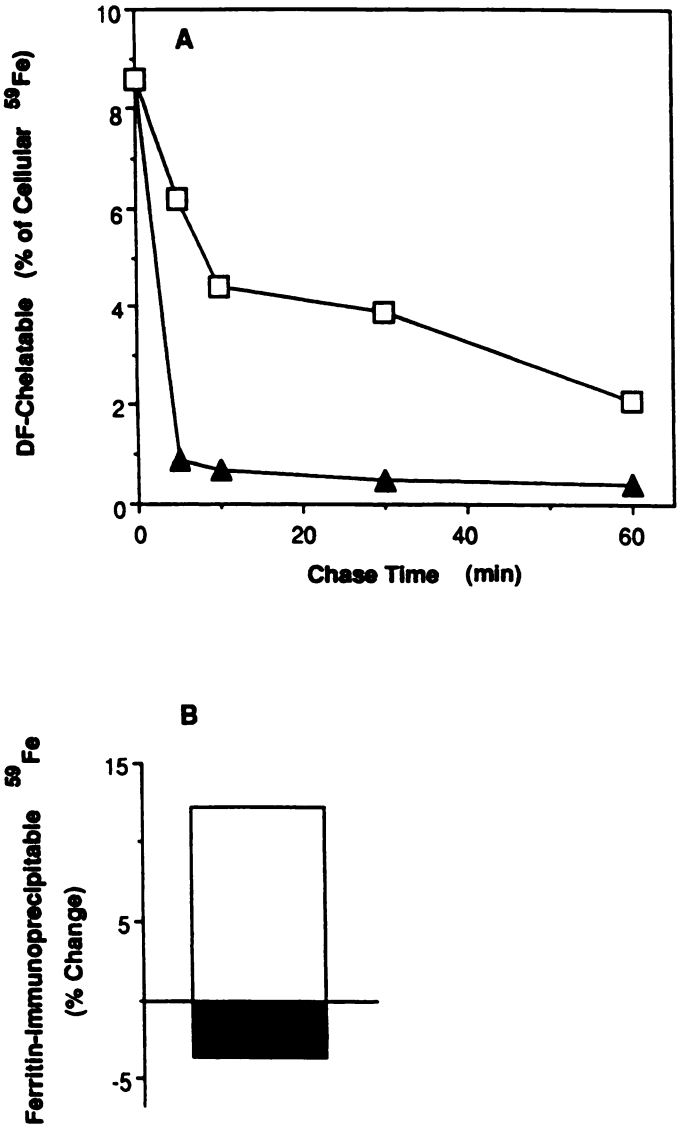

Fig. 3. The DF-chelatable pool of iron is the precursor of the iron that is stored in ferritin. After a 15-min pulse of $^{59}FeCl_3$, the labeling of cultured hepatocytes was chased in the presence (*closed symbols*) or absence (*open symbols*) of 20 mM DF. A, Cellular ^{59}Fe was assayed for the percentage that was chelatable by DF at various times during the chase. B, After a 10-min chase, labeled lysates were assayed for the change in the percentage of the label that was immunoprecipitable as ferritin.

DF treatment of cells (4). Fig. 7 shows that chymostatin similarly inhibited the regeneration of the cellular pool of radioactive iron retained on an Affigel-DF column. The addition of 100 μg/ml chymostatin to the culture medium during the 4-hr incubation reduced by 50% the return of radioactivity to the pool retained on Affigel-DF (Fig. 7A). This agrees closely with the inhibition of the restoration of sensitivity to TBHP that was achieved under the same conditions (Fig. 7B).

Importantly, similar results were observed with the analysis of the immunosupernatants that were obtained by depleting the lysates of ferritin (data not shown). In particular, such immunosupernatants were depleted of chelatable iron after treatment of the cells with DF. Chelatable iron was restored to the immunosupernatants after incubation of DF-pretreated hepatocytes in serum-free medium for 4 hr. Finally, chymostatin inhibited this restoration.

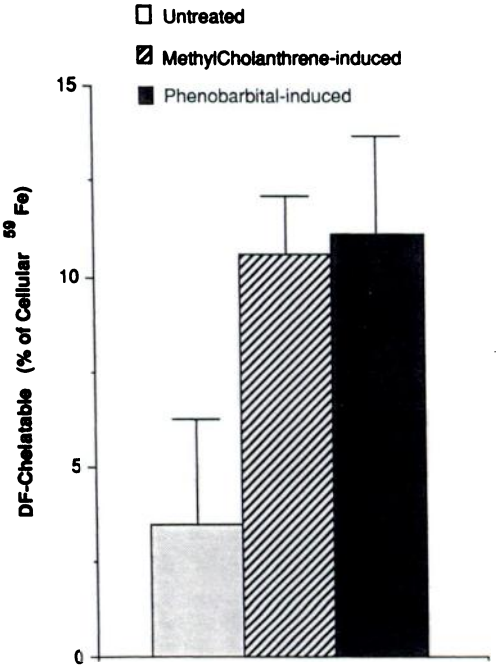

Fig. 4. The labeling of the DF-chelatable pool varies with the metabolic demand for iron. Hepatocytes were prepared from rats that had been treated previously with either methylcholanthrene or phenobarbital or from untreated rats. After overnight culturing, the hepatocytes were labeled with $^{59}FeCl_3$ for 30 min, lysed with Triton X-100, and assayed for DF-chelatable cellular ^{59}Fe. Data represent the results of the analysis of individual cultures obtained from three untreated or three methylcholanthrene-treated rats and triplicate cultures from a single rat treated with phenobarbital.

Discussion

The data presented above establish the existence of a cellular pool of ferric iron that is chelatable by DF and distinct from ferritin. Because most of the iron content of the cultured hepatocyte is stored as ferritin (Figs. 2 and 5A), the dissociation of the DF-chelatable pool from ferritin is critical. The ferric iron pool chelatable by DF could be distinguished from the larger pool of ferritin iron immunochemically as well as kinetically.

The kinetics of labeling of the DF-chelatable pool of ferric iron are distinct from the kinetics of the cellular uptake of iron and the labeling of ferritin (Figs. 2 and 3). Specifically, the iron chelatable by DF is metabolically transient, unlike either the total cellular uptake of iron or its storage in ferritin. Increasing the time that the hepatocytes were labeled with ^{59}Fe decreased the percentage of the total uptake of radioactivity represented by the DF-chelatable pool (Fig. 2). In contrast, ^{59}Fe progressively accumulated in ferritin, in parallel with the increasing cellular uptake of the radiolabeled iron.

Furthermore, with pulse-chase labeling of the hepatocytes, the percentage of total radioactivity represented by the DF-chelatable pool decreased as the percentage represented by ferritin increased (Fig. 3). This result implies that the DF-chelatable iron pool is a precursor of the ferric iron stored in ferritin. Such a conclusion was supported by the fact that treatment of pulse labeled hepatocytes with DF rapidly depleted the cultures of chelatable ^{59}Fe and blocked further incorporation of the label into ferritin. This result cannot be explained by the direct chelation of ferritin iron by DF (Figs. 5B and 6B).

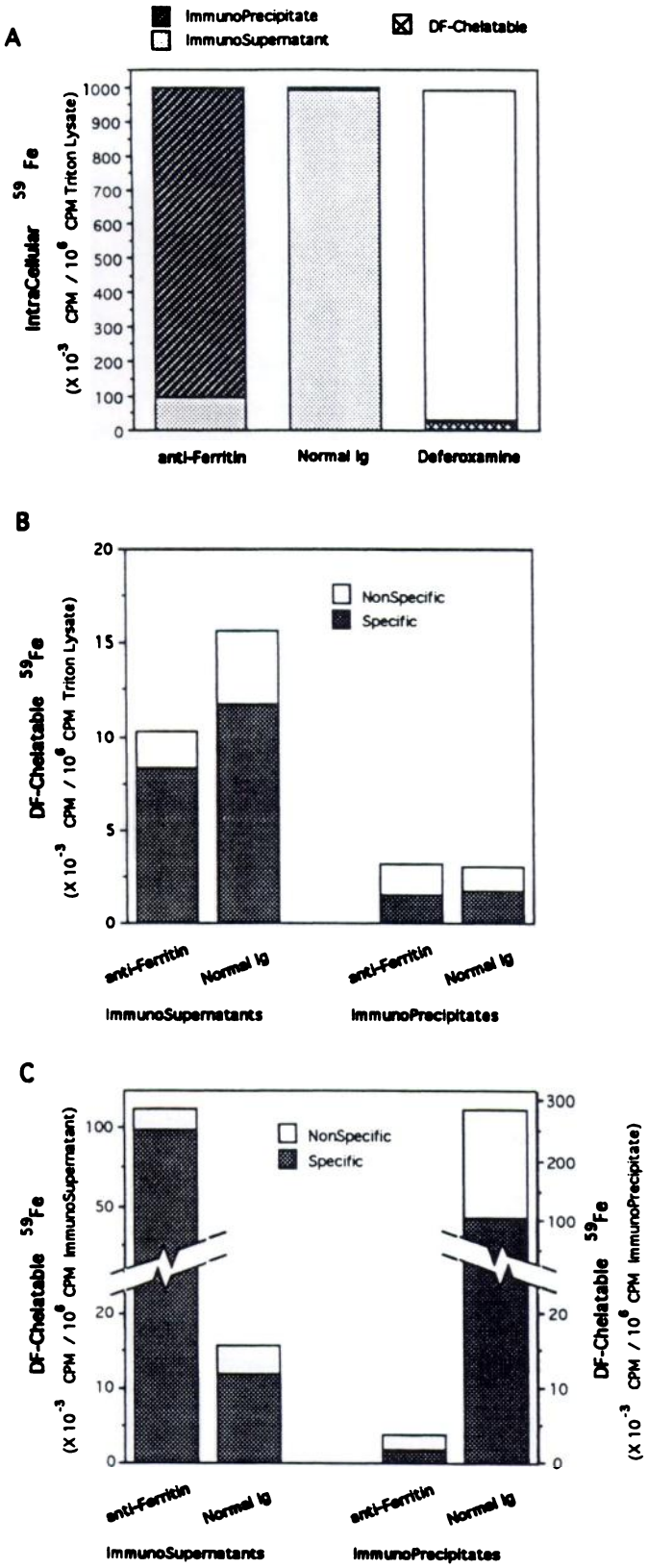

Fig. 5. The DF-chelatable pool of iron is immunochemically distinct from ferritin in steady state labeled hepatocytes. Cultured rat hepatocytes were labeled with ^{59}Fe overnight and then lysed with Triton X-100. Six aliquots of the lysate (each approximately 2×10^6 cpm) were first incubated with a saturating titer of antiferritin immunoglobulin and then immunoprecipitated with an excess of Immunobeads, as described in Experimental Procedures. Alternatively, an additional six aliquots of the lysate were treated first with an equivalent protein concentration of normal immunoglobulin and then with Immunobeads. After a brief centrifugation, the immunosupernatants were separated from the immunoprecipitates, which were washed three times. The resulting six immunosupernatants from each immunoglobulin treatment were analyzed for DF-chelatable ^{59}Fe chromatographically, on Affigel-DF. The total amount of label that bound to the column was corrected for the amount that bound nonspecifically in the presence of soluble DF. Three of the immunoprecipitates were gently agitated in the presence of 10 mM DF. After 1 hr at 4°, the Immunobeads were centrifuged, and the amount of ^{59}Fe released from the immunoprecipitate was determined. As a control, an additional three immunoprecipitates from each of the immunoglobulin treatments were incubated instead with 10 mM sodium mesylate, the desferal (DF) counterion. A, Fractionation of the Triton lysate with antiferritin immunoglobulin, normal immunoglobulin, and DF. B, DF-chelatable ^{59}Fe in the immunosupernatants and immunoprecipitates as a fraction of the Triton lysate. C, DF-chelatable ^{59}Fe in the immunosupernatants and immunoprecipitates as a fraction of the immunosupernatant or immunoprecipitate, respectively.

Similarly, with the human leukemic cell line K562, iron stored in ferritin essentially was not chelatable by DF (14).

The DF-chelatable pool is also distinguishable immunochemically from ferritin (Figs. 5 and 6). Whereas an antiferritin antibody quantitatively depleted cell lysates of ferritin, the resulting immunosupernatants, but not the immunoprecipitates, were enriched in DF-chelatable iron. In contrast, the control treatment of labeled cell lysates with normal (nonspecific) immunoglobulin enriched the immunoprecipitates, but not the immunosupernatants, for DF-chelatable iron.

Thus, enrichment for DF-chelatable iron was achieved by separating this iron pool from ferritin, by immunoprecipitating either ferritin or the chelatable pool itself. Furthermore, the degree of enrichment for chelatable iron correlated with the percentage of cellular label that was immunoprecipitable as ferritin. Thus, a greater enrichment was obtained upon treatment of lysates from steady state labeled, as opposed to pulse labeled, cells. This result correlates with the observation that ferritin represents a greater percentage of the total cellular radioactivity after steady state labeling, as opposed to pulse labeling.

The ability of the control incubation with normal immunoglobulin to enrich immunoprecipitates for chelatable iron was a surprise. Interestingly, a similar percentage of the Triton lysate was immunoprecipitated by incubation with the Immunobeads (goat anti-rabbit immunoglobulin) alone (data not shown). We speculate that a rat cell surface antigen recognized by goat anti-rabbit immunoglobulin cross-reacts with a protein to which the DF-chelatable iron is bound.

The DF-chelatable iron pool identified in this paper most likely represents a metabolically common pool of ferric iron that is rapidly in transit through the cell. Extracellular iron enters this pool upon its uptake by the cell. In turn, the iron in this pool is utilized for heme synthesis or stored in ferritin, depending upon the metabolic needs of the cell. Ferric iron can be released into this common pool, at least in part, as a result of the autophagic degradation of ferritin. The pool we have identified would be consistent with the intracellular intermediates in iron transport that have been studied primarily in hemoglobin biosynthesis by reticulocytes (15–17). Its relationship to previously identified (18–21) sources of nonheme nonferritin iron remains to be determined.

The DF-chelatable iron pool also represents a source of ferric iron required for oxidative cell injury. Treatment of cultured

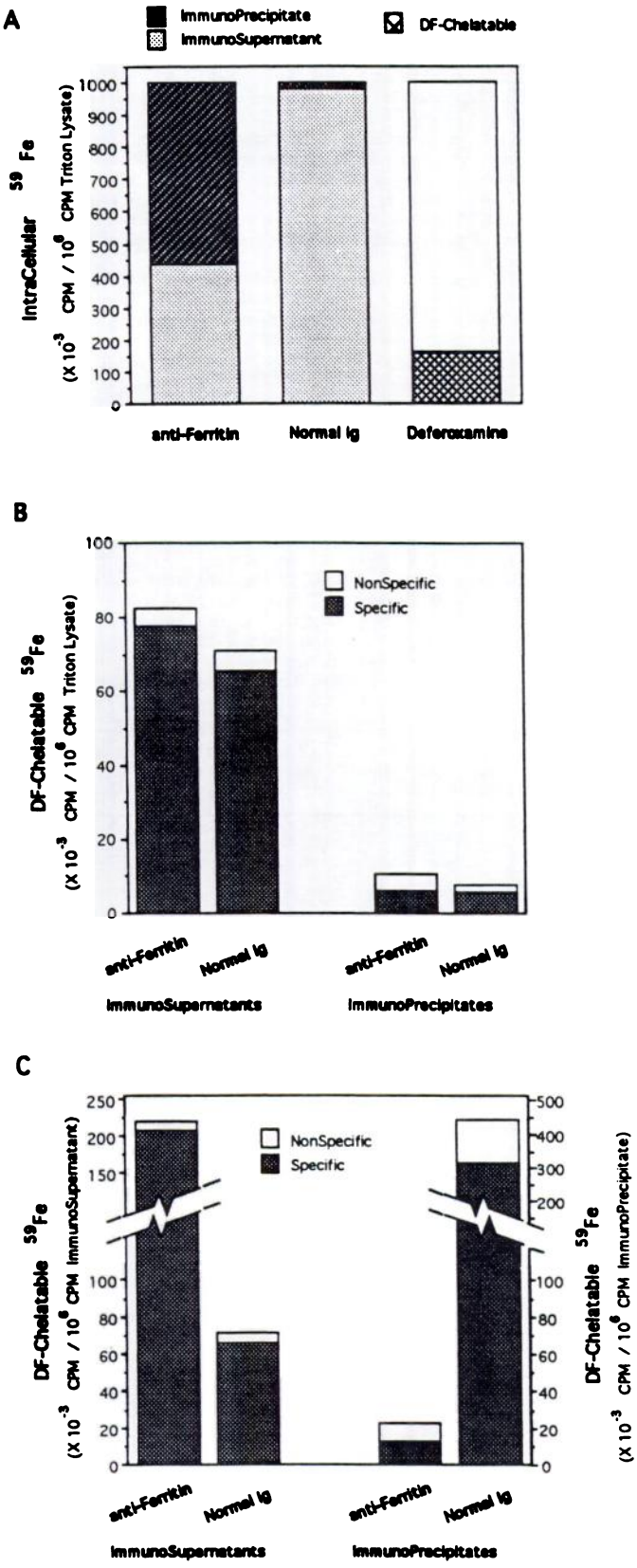

Fig. 6. The DF-chelatable pool of iron is immunochemically distinct from ferritin in pulse labeled hepatocytes. Cultured hepatocytes were labeled with a 20-min pulse of ^{59}Fe and then lysed with Triton X-100. Aliquots of the Triton lysate (approximately 10^5 cpm) were treated as described for Fig. 5.

hepatocytes with DF depletes the ferric iron pool in parallel with the prevention of oxidative cell injury (Fig. 7). Both the DF-chelatable iron pool and the sensitivity to an oxidative stress are restored by incubation of the cells in the absence of exogenous iron (Fig. 7). In addition, inhibition of protein degradation by chymostatin inhibits both the restoration of the

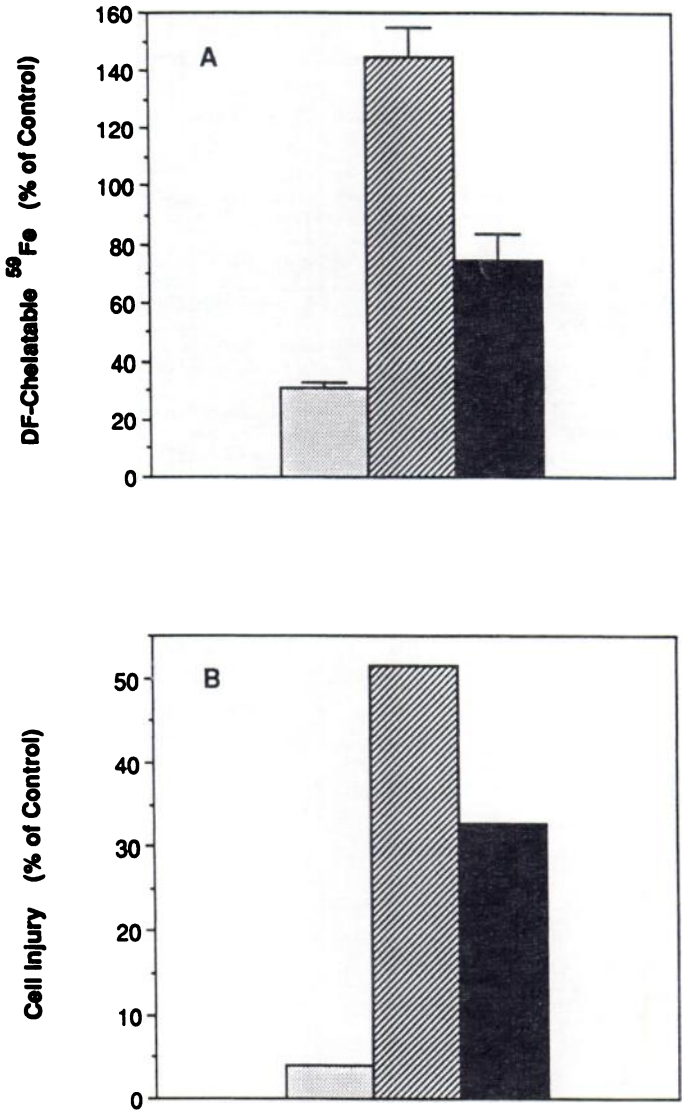

Fig. 7. The DF-chelatable pool of iron is associated with oxidative cell injury. Cultured hepatocytes were incubated with 20 mM DF for 1 hr and then assayed for the effect of this treatment upon both DF-chelatable intracellular iron and susceptibility to oxidative cell injury. Alternatively, additional cultures were washed and allowed to recover from the DF treatment by incubation in the absence of DF, but in the presence or absence of chymostatin (100 μg/ml), for 4 hr before assaying as described above. Control cultures were not treated with DF. Data are presented relative to values from the appropriate control cultures, which were assayed either at the start or at the conclusion of the 4-hr recovery period. Although the total intracellular ^{59}Fe decreased approximately 10% during the 4-hr incubation, the percentage of label that was chelatable remained equivalent in both sets of control cultures. DF-treated cultures were assayed before (□) or after (▨) recovery or after recovery in the presence of chymostatin (■). A, DF-chelatable iron. Cultures, labeled overnight with ^{59}Fe, were treated as described above, lysed, and assayed for DF-chelatable cellular ^{59}Fe. B, Oxidative cell injury. First the treated cultures were incubated with 0.5 mM TBHP for 1 hr and then the extent of cell injury was determined.

DF-chelatable iron pool and the sensitivity of the hepatocytes to an oxidative stress (Fig. 7).

It has been suggested that an oxidative stress may reductively release iron from ferritin and thereby promote oxidative cell injury (6–8). The data presented above do not exclude the possibility that such a mechanism may contribute to the iron pool required for oxidative injury. However, it deserves emphasis that this requisite iron pool also exists independently of an oxidative stress. In other words, an oxidative stress is not necessary for the generation of this pool of iron. Rather, the requisite pool pre-exists as a consequence of the physiologic metabolism of iron as iron moves through the cell.

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