

# Chloramphenicol-Induced Mitochondrial Dysfunction Is Associated With Decreased Transferrin Receptor Expression and Ferritin Synthesis in K562 Cells and Is Unrelated to IRE-IRP Interactions

LORENE M. LEITER,<sup>1</sup> HEMANT S. THATTE,<sup>2</sup> CHUKWUKA OKAFOR,<sup>1</sup>  
 PETER W. MARKS,<sup>1</sup> DAVID E. GOLAN,<sup>2</sup> AND KENNETH R. BRIDGES<sup>1,2\*</sup>

<sup>1</sup>Department of Medicine, Harvard Medical School, Division of Hematology, Brigham and Women's Hospital, Boston, Massachusetts

<sup>2</sup>Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Division of Hematology, Brigham and Women's Hospital, Boston, Massachusetts

Chloramphenicol is an antibiotic that consistently suppresses the bone marrow and induces sideroblastic anemia. It is also a rare cause of aplastic anemia. These toxicities are thought to be related to mitochondrial dysfunction, since chloramphenicol inhibits mitochondrial protein synthesis. We hypothesized that chloramphenicol-induced mitochondrial impairment alters the synthesis of ferritin and the transferrin receptor. After treating K562 erythroleukemia cells with a therapeutic dose of chloramphenicol (10 µg/ml) for 4 days, there was a marked decrease in cell surface transferrin receptor expression and de novo ferritin synthesis associated with significant decreases in cytochrome *c* oxidase activity, ATP levels, respiratory activity, and cell growth. Decreases in the transferrin receptor and ferritin were associated with reduced and unchanged message levels, respectively. The mechanism by which mitochondrial dysfunction alters these important proteins in iron homeostasis is not clear. A global decrease in synthetic processes seems unlikely, since the expression of the cellular adhesion proteins VLA4 and CD58 was not significantly decreased by chloramphenicol, nor were the message levels of β-actin or ferritin. The alterations were not accompanied by changes in binding of the iron response protein (IRP) to the iron-responsive element (IRE), although cytosolic aconitase activity was reduced by 27% in chloramphenicol-treated cells. A disturbance in iron homeostasis due to alterations in the transferrin receptor and ferritin may explain the hypochromic-microcytic anemia and the accumulation of nonferritin iron in the mitochondria in some individuals after chloramphenicol therapy. Also, these studies provide evidence of a link between mitochondrial impairment and iron metabolism in K562 cells. *J. Cell. Physiol.* 180:334–344, 1999. © 1999 Wiley-Liss, Inc.

Chloramphenicol, first isolated from *Streptomyces venezuela* in 1947 (Ehrlich et al., 1947; Bartz, 1948), is a potent antibiotic effective against a large number of organisms. Unfortunately, it is also linked with serious toxicities, including bone marrow suppression, sideroblastic anemia, and, more infrequently, with fatal aplastic anemia (Yunis and Salem, 1980). Due to its severe side effects, chloramphenicol is seldom prescribed in the United States today except for the treatment of life-threatening infections. However, due to its effectiveness and low cost, it continues to be used extensively worldwide, particularly in developing countries (Holt et al., 1997). Elucidating the mechanism by which chloramphenicol causes toxicity is essential in order to design safer strategies for its use. In addition, an understanding of the pathways by which the drug

causes aplastic and sideroblastic anemia could provide valuable insight into the pathogenesis of these diseases, the causes of which, in most cases, have not been determined.

Over the past 40 years, significant progress has been made in understanding the pathogenesis of chloram-

Contract grant sponsor: NIH; Contract grant numbers: R01HL-15157, R01HL-32854, and R01-HL45794-07; Contract grant sponsor: NIH National Research Science; Contract grant sponsor: NHLBI; Contract grant number: K08-HL-03235.

\*Correspondence to: Kenneth R. Bridges, M.D., Division of Hematology/Oncology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. E-mail: bridges@calvin.bwh.harvard.edu

Received 2 December 1998; Accepted 13 April 1999

phenicol-induced hematological toxicity. Much attention has been focused on the role of mitochondrial injury. Chloramphenicol limits bacterial growth by inhibiting the 50S ribosomal subunit and consequently protein synthesis. Unfortunately, the similarities between mitochondria and bacteria render these eukaryotic organelles susceptible to this inhibition (Hayes, 1994). Mitochondrial DNA codes for 13 polypeptides, which are subunits of enzyme complexes involved in electron transport and oxidative phosphorylation, including NADH dehydrogenase, ATPase, and cytochrome *c* oxidase (Attardi, 1993). Nuclear DNA does not code for these subunits, leaving the cell completely dependent on mitochondria for their synthesis. It is generally agreed that chloramphenicol causes bone marrow dysfunction, including defective heme synthesis, secondary to its effect on mitochondrial metabolism, although the pathway by which this occurs is not known.

In this study, we investigated the effect of chloramphenicol on mitochondrial function in K562 cells, a human erythroleukemia cell line frequently used as a model for red blood cell precursors. We also examined the drug's effect on the synthesis of two key proteins in iron metabolism: the transferrin receptor, which mediates iron uptake; and ferritin, the iron storage protein. Alterations in these proteins could affect iron uptake and safe storage of iron. Thus, these studies may help elucidate the mechanisms by which chloramphenicol: 1) impairs erythropoiesis and heme synthesis, which processes are highly dependent on iron; and 2) causes accumulation of nonferritin iron in the mitochondria of erythroid precursors in individuals who develop sideroblastic anemia.

Chloramphenicol was found to substantially reduce cell growth, cytochrome *c* oxidase activity, ATP synthesis, aconitase activity, ferritin synthesis, and cell surface transferrin receptor expression. Interestingly, the alterations in ferritin synthesis and transferrin receptor expression were not associated with changes in the binding affinity of the iron response protein (IRP) to the iron-responsive element (IRE). Numerous studies in nonerythroid cell lines have shown that interactions of IRPs with IREs on ferritin and transferrin receptor RNA control iron metabolism in these cells (Harford et al., 1994). However, there is less evidence that this mode of regulation is as important in erythroid cells (Ponka, 1997).

Although chloramphenicol penetrates all cells and even crosses the blood-brain barrier, its side effects are limited to the bone marrow and particularly to developing normoblasts. These cells have an extremely high requirement for iron, and chloramphenicol's effect on key proteins in iron homeostasis in these cells may explain their sensitivity to the drug's effects. In addition, these findings suggest a relationship between mitochondrial dysfunction and iron metabolism in K562 cells.

## MATERIALS AND METHODS

### Cell culture and treatments

Human K562 erythroleukemia cells (ATCC, Rockville, MD) were maintained at a concentration of  $1 \times 10^6$  cells/ml in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% newborn

calf serum (BioWhittaker) at 37°C, 5% CO<sub>2</sub>. Prior to all experiments, cells were harvested and resuspended in complete medium at a concentration of  $2 \times 10^5$  cells/ml. Treatments included: 1) 10, 25, or 40 µg/ml water-soluble chloramphenicol (Sigma Chemical Co., St. Louis, MO) for 2, 4, or 8 days; 2) 200 µg/ml ferric ammonium citrate for 24 h; and 3) 100 µM desferrioxamine (deferrioxamine mesylate USP from CIBA, Summit, NJ) for 24 h. Iron uptake in ferric ammonium citrate-treated cells was confirmed by Prussian blue staining of fixed cells. Cells were counted on a hemacytometer and viability determined by trypan blue exclusion. Cell cycle profiles were determined by propidium iodide staining followed by FACS analysis using standard procedures (protocol accompanying propidium iodide from Boehringer Mannheim Corp., Indianapolis, IN).

### Isolation of mitochondrial fractions

Cells ( $1-2 \times 10^7$ ) were harvested and resuspended in 0.5 ml ice-cold hypotonic buffer containing 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, pH 7.5, and 1 mM phenylmethylsulfonylfluoride (PMSF), transferred to 1.5 ml Eppendorf tubes, and maintained on ice for 10 min. Cells were then gently homogenized manually 20 times with a 6-cm polypropylene pestle (Kontes pellet pestle, Fisher Scientific, Pittsburgh, PA) and mixed with 0.7 ml mitochondrial solubilization buffer (0.525 M D-mannitol, 175 mM sucrose, 12.5 mM EDTA, and 12.5 mM Tris-HCl, pH 7.5) (Higuchi and Linn, 1995). The mitochondrial fraction was obtained by centrifugation at 1,000 rpm in a microfuge for 5 min at 4°C (to remove the nuclear fraction) followed by centrifugation of the supernatant at 10,000 rpm for 10 min at 4°C.

### Cytochrome *c* oxidase (EC 1.9.3.1) activity

Mitochondrial fractions were resuspended in 200 µl ice-cold 10 mM potassium phosphate buffer, pH 7.0. Protein was quantitated colorimetrically with Coomassie Brilliant Blue G-250 dye (Bio-Rad Laboratories, Hercules, CA). Cytochrome *c* oxidase was determined by a modification of the assay of Smith (1955). Mitochondrial suspensions (5 µg) containing cytochrome *c* oxidase were added to cuvettes containing 2.8 ml 10 mM potassium phosphate buffer and 0.2 ml reduced 1% w/v cytochrome *c* (from horse heart, Sigma). Cytochrome *c* was reduced by adding 3-5 mg L-ascorbic acid to 10 ml 1% cytochrome *c* (in 10 mM potassium phosphate buffer) and dialyzing overnight at 4°C. Cytochrome *c* oxidase activity was determined by spectrophotometric analysis by measuring a decrease in optical density at 550 nm after 10 min at 37°C. Activity was expressed as  $\Delta$  O.D. 550/min/mg protein.

### ATP determination

Cells ( $2 \times 10^7$ ) were harvested and resuspended in 1 ml Tris-EDTA buffer (4 mM EDTA, 100 mM Tris, pH 7.75). After removing aliquots for protein determination, cells were boiled for 3 min and protein precipitates were removed by centrifugation at  $10,000 \times g$  for 60 sec. Supernatants were transferred to a fresh tube and maintained on ice until ATP measurement. Cellular ATP was determined by adding 100 µl luciferase-luciferin reagent (Sigma) (10 mg/ml) to the supernatant (10 µl) in 390 µl Tris-EDTA buffer and immediately placed into a biolumi-

nometer (Monolight 2010, Analytical Luminescence Laboratory, Sparks, MD), integrating the light signal for 20 sec. ATP concentrations were calculated from a standard curve using the log of ATP standards vs. the log of bioluminescent units. The data were expressed as nmole ATP/mg protein in cell lysates.

### Mitochondrial respiration

Mitochondrial metabolic function *in vivo* was assessed with the use of a MitoTracker Orange CM-H<sub>2</sub>TMRos dye (Molecular Probes, Eugene, OR) which, upon entering an actively respiring cell, is oxidized to a fluorescent mitochondrion-selective probe and sequestered in the mitochondria. A MitoTracker Green FM probe (Molecular Probes), which fluoresces once it accumulates in the lipid environment of mitochondria, was used as a measure of mitochondrial number. Cells ( $5 \times 10^6$ ) were harvested and resuspended in RPMI 1640 medium containing 50 nM of each dye and incubated at 37°C for 15 min in a waterbath. Cells were washed three times with phosphate-buffered saline (PBS) and fixed in 3.7% formaldehyde (in PBS) for 15 min. After washing twice with PBS, cells were cytospun onto glass coverslips that were mounted with aqueous Gel/Mount media (Fisher Scientific) onto glass slides and sealed with nail polish. Cells were observed using a Zeiss Axioskop epifluorescence microscope equipped with differential interference contrast optics. The cells were imaged at 250–1,000 $\times$  magnification using a CCD camera (Model CH250, Photometrics Ltd., Tucson, AZ) cooled to  $-40^\circ\text{C}$ . Pseudocolor fluorescence images were digitized. The cell fluorescence was measured using an image processor (Metamorph, Universal Imaging, West Chester, PA) and expressed in arbitrary units as fluorescence counts per cell.

### Transferrin receptor, CD58, and VLA4 cell surface expression

**Transferrin receptor.** Cells ( $10^6/\text{ml}$ ) were incubated with 10  $\mu\text{M}$  fluorescein isothiocyanate (FITC)-conjugated holo-transferrin (FITC-TF; Molecular Probes) in PBS at 4°C for 90 min. Cells were then washed twice with Hanks' balanced salt solution (HBSS; Sigma), fixed in 3.7% formaldehyde in PBS, and maintained at 4°C for quantitative fluorescence microscopy measurements.

**CD58 and VLA4.** In order to test the specificity of the effect of chloramphenicol on the transferrin receptor, we also quantitated the cell surface expression of two cellular adhesion molecules, CD58 and VLA4. Cells ( $10^6/\text{ml}$ ) were incubated with phycoerythrin (PE)-conjugated mouse monoclonal anti-human CD58 (LFA-3, IgG) (Becton Dickinson Immunochemicals, San Jose, CA) or FITC-conjugated mouse monoclonal anti-human  $\alpha 4\beta 1$  (CD49d, VLA4, IgG) (Calbiochem, La Jolla, CA) (50  $\mu\text{g}/\text{ml}$ ) in complete medium for 60 min at room temperature with frequent, gentle mixing. Cells were washed twice with complete medium and the cell surface expression of the transferrin receptor, CD58, and VLA4 was measured by quantitative fluorescence microscopy as outlined above. Pseudocolor fluorescence images were printed on a Phaser IISDX color printer (Tektronix, Beaverton, OR).

### Ferritin synthesis

Cells ( $2 \times 10^7$ ) were harvested and resuspended in 5 ml RPMI 1640 medium without methionine or serum and incubated for 15 min at 37°C. Cells were then gently pelleted and resuspended in the same medium containing 200  $\mu\text{Ci}$   $^{35}\text{S}$ -methionine and respective treatments. After 1 h at 37°C, cells were harvested and washed three times with cold PBS. Cell pellets were resuspended in 200  $\mu\text{l}$  cold lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.2% NP40, and 1 mM PMSF. After 20 min on ice, samples were spun at 10,000 rpm in a microfuge for 10 min at 4°C. Supernatants were immunoprecipitated with rabbit anti-human ferritin antibody (Boehringer Mannheim Corp.) for 1 h at 4°C on a rotator followed by incubation with 20  $\mu\text{l}$  immobilized Protein A (Pierce, Rockford, IL) for 1 h at 4°C. Protein A beads were pelleted by centrifuging samples at 1,500 rpm for 5 min and washed three times with lysis buffer containing 0.5 M NaCl (first wash), 0.1% sodium lauryl sulfate (SDS) (second wash) and lysis buffer alone, for 10 min each, at 4°C. Beads were then resuspended in 10  $\mu\text{l}$  Laemmli buffer, boiled for 5 min, and run on a 15% acrylamide gel. The gel was fixed in 25% methanol and 7% acetic acid (v/v) for 1 h, treated with fluorographic reagent (Amersham, Pharmacia Biotech, Piscataway, NJ) for 30 min, dried, and exposed to X-Omat Kodak film for 24 h at  $-80^\circ\text{C}$ . Ferritin bands on the film were quantitated using a densitometer (Molecular Dynamics, Sunnyvale, CA).

### Total protein synthesis

The amount of  $^{35}\text{S}$ -methionine incorporated into protein was assessed to determine whether total protein synthesis was affected by chloramphenicol. Lysates (5  $\mu\text{g}$ ) used for the ferritin immunoprecipitations were mixed with an equal volume (5  $\mu\text{l}$ ) of 10 mg/ml bovine serum albumin (BSA) and pipetted onto glass fiber filters. Protein was precipitated onto the filters by addition of 10% trichloroacetic acid (TCA) and incubation for 30 min at 4°C. After two 5-min washes with TCA, filters were washed with 95% ethanol, dried, and quantitated in a scintillation counter.

### Transferrin receptor, H-ferritin, and L-ferritin mRNA

RNA isolation, electrophoresis, transfer, and Northern hybridization procedures were performed according to the protocol included with Stratagene's RNA isolation kit (La Jolla, CA). Briefly, 25  $\mu\text{g}$  of RNA was run on a 1% agarose-formaldehyde gel and transferred to a Hybond-N<sup>+</sup> nylon filter (Amersham) through capillary transfer. RNA was fixed to the membrane by baking for 2 h at 80°C. Hybridization probes for the transferrin receptor, H-ferritin, L-ferritin, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and  $\beta$ -actin were radiolabeled with  $^{32}\text{P}$ -dCTP using Stratagene's Prime-It<sup>®</sup> Random Primer Labeling Kit and purified on NucTrap probe purification columns (Stratagene). cDNA was obtained from a 700-bp *Pst* I fragment of pCDTR-1 (transferrin receptor, McClelland et al., 1984); a 600 *Pst* I fragment of pSV2H-ferritin (H-ferritin, Constanzo et al., 1986); and a 670-bp *Pst* I fragment of SV2L-ferritin (L-ferritin, Dente et al.,



1985). RNA levels on the film were quantitated using a densitometer (Molecular Dynamics).

### Cytosolic aconitase activity

Aconitase activity was measured by a modification of a previously described method (Davies and Scopes, 1981). Cells were lysed in 500  $\mu$ l ice-cold buffer containing 10 mM HEPES, pH 7.5, 40 mM KCl, 5% glycerol, 3 mM MgCl<sub>2</sub>, 0.3% Nonident-p40, and 1 mM PMSF. After 5 min on ice, cells were centrifuged at 14,000 rpm for 30 min at 4°C. Cell lysates (50  $\mu$ g) were added to cuvettes containing 0.2 mM *cis*-aconitate in a 1-ml assay buffer (containing 50 mM Tris-HCl, pH 7.2, 100 mM NaCl, and 0.02% BSA). Activity was measured spectrophotometrically by the disappearance of *cis*-aconitate at 240 nm after 10 min. Activity was expressed as  $\mu$ M substrate converted/mg protein/min.

### Gel retardation assay

IRE-protein interactions were assayed by a modification of the method of Leibold and Munro (1988). Radiolabeled IRE probes were made with an RNA transcription kit (Stratagene), [ $\alpha$  <sup>32</sup>P] rUTP (NEN, Life Science Products, Boston, MA), and a 28-bp oligonucleotide encoding the human ferritin IRE. The IRE oligonucleotide was obtained by digesting pSPT-fer plasmid (the kind gift of Dr. Kuhn; see Mullner et al., 1989) with Bam HI. Excess probe (50,000 cpm) was incubated with 20  $\mu$ g freshly prepared cell lysate (prepared as in the aconitase assay method above) on ice for 30 min. RNase T1 (1 U per reaction) and heparin (5 mg/ml) were added sequentially for 10 min each. An additional set of reactions also contained 2% 2-mercaptoethanol, which causes full activation of IRP binding to the IRE probe and thus allows determination of total cellular IRP levels. IRE-protein complexes were resolved on a 10% nondenaturing polyacrylamide gel (29:1 acrylamide, bis-acrylamide) containing 1X TBE (tris-borate-EDTA) buffer.

### Statistical analysis

ATP and mitochondrial respiration data were statistically analyzed with the unpaired Student's *t*-test. Aconitase activity was analyzed by one-way analysis of variance (ANOVA) and means were categorized by Fisher's LSD test. Significance was assumed at *P* < 0.05.

## RESULTS

### Chloramphenicol decreases cell proliferation

Figure 1A displays the effect of various doses of chloramphenicol on K562 cell growth after 2, 4, 6, and 8 days of treatment. These doses were chosen because they reflect the range of peak serum concentrations found in patients after chloramphenicol administration (10–25  $\mu$ g/ml), as well as toxic concentrations found more infrequently (40  $\mu$ g/ml). All three levels of chloramphenicol inhibited cellular proliferation by approximately 51% on day 2, 54% on day 4, 55% on day 6, and 62% on day 8. Cell cycle analysis revealed that growth inhibition was associated with 1) a gradual increase in the percent of cells in G1, averaging 37% higher than untreated controls by day 8 of treatment; and 2) a gradual decrease in the percent of cells in S-phase, averaging 44% less by day 8 of treatment compared to

untreated controls (data not shown). Apoptotic cells were also present on day 8 of chloramphenicol treatment, but not on day 4.

### Chloramphenicol impairs mitochondrial function

**Cytochrome *c* oxidase activity.** Mitochondria were isolated and cytochrome *c* oxidase activity was measured in order to confirm chloramphenicol's previously reported effect of inhibiting mitochondrial metabolic function (Fig. 1B). Mitochondria synthesize 13 proteins of the inner mitochondrial membrane, including three subunits of cytochrome *c* oxidase. We expected that chloramphenicol's suppression of mitochondrial protein synthesis would depress cytochrome *c* oxidase activity. Activity was measured after treatment with 10, 25, or 40  $\mu$ g chloramphenicol/ml of culture medium for 2, 4, and 8 days. Cytochrome *c* oxidase activity fell sharply with all doses of chloramphenicol by day 2, with activity of treated groups averaging only about 24% that of untreated controls. Activity declined further with time, dipping to only about 11% that of untreated groups by day 4 and 3% by day 8.

We directly assessed inhibition of mitochondrial protein synthesis by labeling cells for 2 h with <sup>35</sup>S-methionine while inhibiting cytoplasmic protein synthesis with cycloheximide. After isolating mitochondrial fractions and resolving mitochondrial proteins on a 10–20% SDS polyacrylamide gel followed by autoradiography, only faint mitochondrial protein bands were visible in chloramphenicol-treated cells (data not shown).

Since the 10  $\mu$ g/ml dose of chloramphenicol suppressed both cell growth and cytochrome *c* oxidase activity to the same extent as the higher doses, we chose the 10  $\mu$ g/ml dose for all subsequent experiments. Also, since people normally take the drug for several days, we maintained the cells in chloramphenicol for 4 days in all subsequent experiments.

**Total cellular ATP.** Total cellular ATP measurements assessed the effect of chloramphenicol on mitochondrial function (Fig. 1C). After 4 days of chloramphenicol (10  $\mu$ g/ml) treatment, ATP levels were significantly lower in chloramphenicol-treated cells (53.5  $\pm$  5.9 nM/mg protein) (mean  $\pm$  SEM) compared to untreated controls (119.4  $\pm$  13.7 nM/mg protein). Thus, energy production in chloramphenicol-treated cells was impaired as indicated by ATP levels of only 45% of control values.

**Mitochondrial respiration.** To assess mitochondrial respiratory activity, we used MitoTracker Orange CM-H<sub>2</sub>TMRos, which, upon entering an actively respiring cell, is oxidized to a cationic fluorescent compound. Thus, the fluorescence intensity is proportional to the degree of oxygen utilization by the mitochondria. The positive charge of these probes allows them to localize to the negatively charged mitochondrial membrane and bond with thiol groups on proteins and peptides to form aldehyde-fixable conjugates. (The details of the probe's action are described in literature accompanying this Molecular Probes product). We incubated K562 cells for 15 min with this dye to assess *in vivo* mitochondrial respiratory activity. Cells were fixed in 3.7% formaldehyde and immediately visualized using an epi-

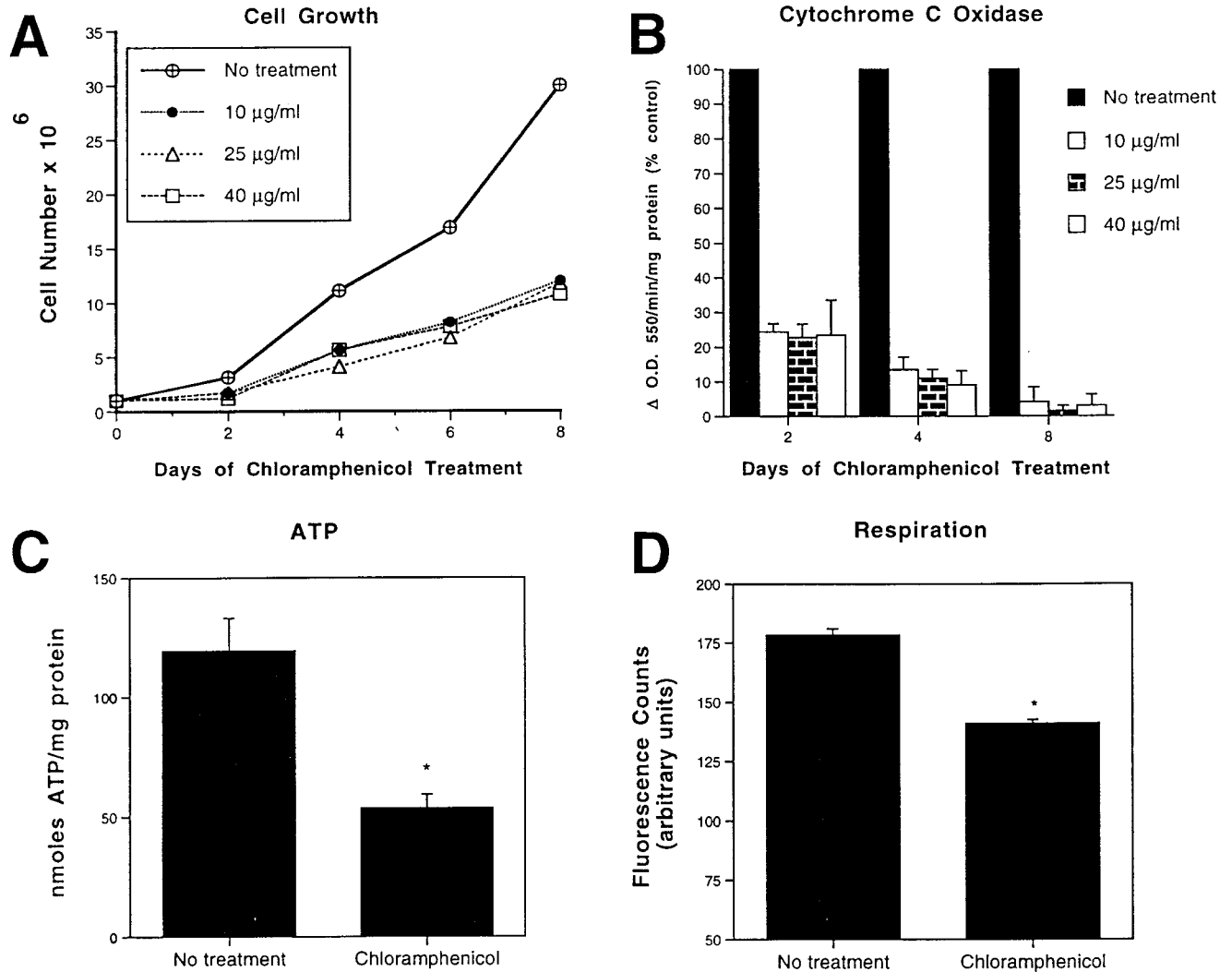


Fig. 1. **A:** K562 cell growth after treatment with chloramphenicol (10, 25, or 40  $\mu\text{g/ml}$ ) for 2, 4, 6, and 8 days. The cell counts are from one set of experiments in duplicate and are virtually identical to counts in all subsequent experiments. Cells were counted on a hemacytometer chamber after staining in trypan blue. **B:** Cytochrome *c* oxidase activity in K562 cells after treatment with chloramphenicol (10, 25, or 40  $\mu\text{g/ml}$ ) for 2, 4, and 8 days. Activity was measured by adding mitochondrial suspensions to cuvettes containing reduced cytochrome *c* and following the optical density (550) decrease over 10 min at 37°C. Data are expressed as a percent of untreated controls and represent the mean  $\pm$  SEM ( $n = 3$ , days 2 and 4, and  $n = 2$ , day 8). (Note: typical untreated control values average approximately 1.4  $\Delta$  O.D. 550/min/mg protein.) **C:** Total ATP in K562 cells treated with chloramphenicol (10  $\mu\text{g/ml}$ ) for 4 days. Cellular ATP levels were

measured by bioluminometry. Luciferase-luciferin reagent was added to supernatants after boiling to precipitate protein. Values are the mean  $\pm$  SEM,  $n = 4$ . \*Represents a significant difference by unpaired Student's *t*-test analysis with  $P < 0.01$ . **D:** Mitochondrial respiration in K562 cells treated with chloramphenicol (10  $\mu\text{g/ml}$ ) for 4 days. Cells were incubated for 15 min at 37°C with MitoTracker Orange CM-H<sub>2</sub>TMRos which, upon entering an actively respiring cell, is oxidized to a fluorescent probe and sequestered in the mitochondria. Cells were observed using a Zeiss Axioskop epifluorescence microscope. The fluorescence in each cell was measured (in arbitrary units) using an image processor (see Materials and Methods). Values are the mean  $\pm$  SEM,  $n = 141$  (untreated) and  $n = 181$  (chloramphenicol-treated). \*Represents a significant difference by unpaired Student's *t*-test analysis with  $P < 0.0001$ .

fluorescence microscope. Fluorescence was quantitated in each cell by an image processor (see Materials and Methods). Figure 1D shows that chloramphenicol significantly decreased fluorescence, indicating that the drug reduced mitochondrial respiratory activity in these cells. This decrease indicated impaired respiratory activity and not fewer mitochondria per cell, since no decrease in fluorescence was found after labeling with MitoTracker Green FM fluorophore. Green FM specifically accumulates in the mitochondrial lipid

membrane and thus is a probe for mitochondrial number in each cell.

#### Chloramphenicol decreases cell surface expression of the transferrin receptor, but not CD58 and VLA4

Cell surface expression of the transferrin receptor, CD58, and VLA4 was analyzed by quantitative fluorescence microscopy after incubating cells with FITC-conjugated transferrin, PE-conjugated anti-CD58 antibody,

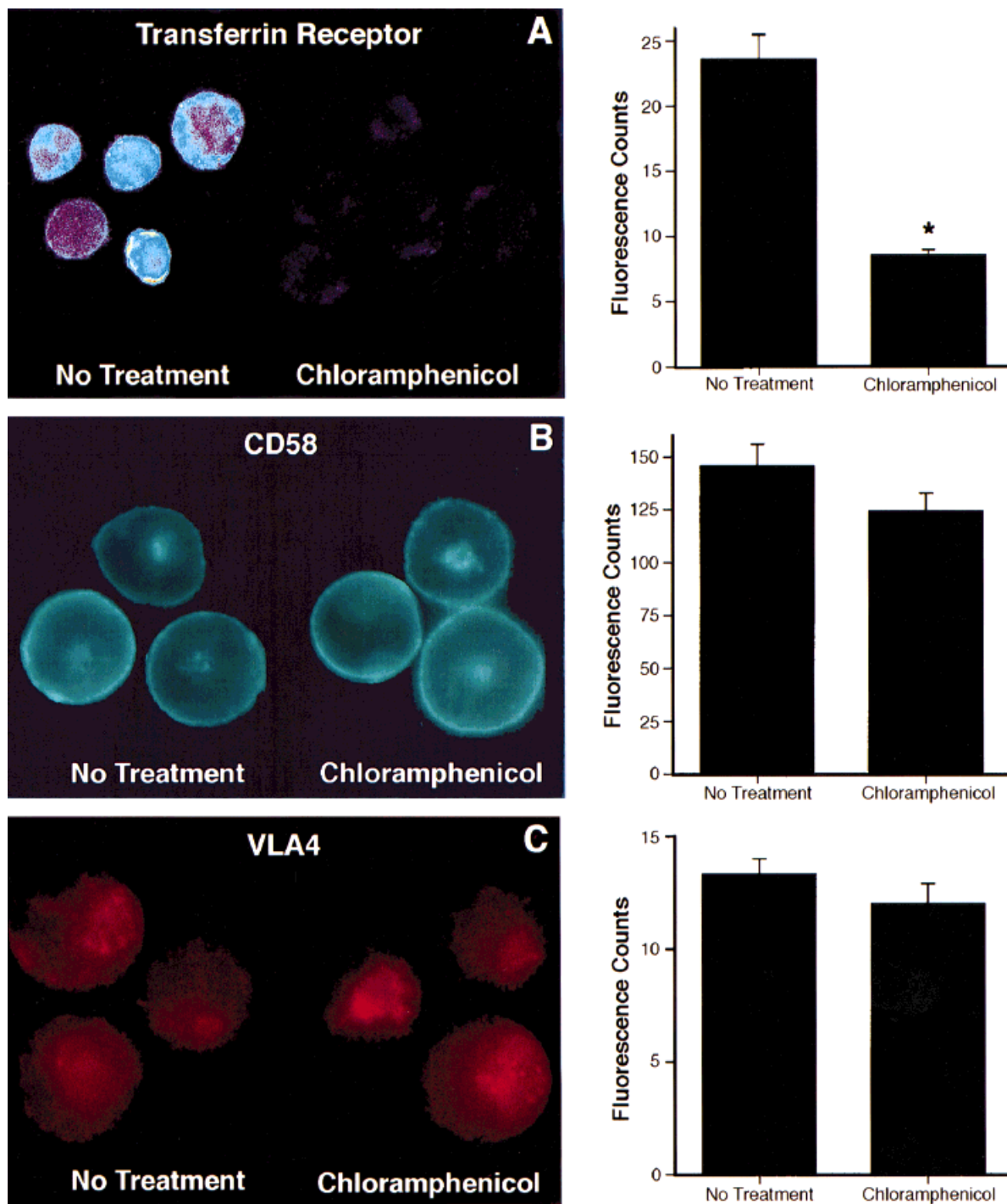


Fig. 2. Transferrin receptor, CD58, and VLA4 cell surface expression in K562 cells treated with chloramphenicol (10  $\mu\text{g/ml}$ ) for 4 days. Cells were incubated with **A**: FITC-TF; **B**: PE-conjugated anti-human CD58; or **C**: FITC-conjugated anti-human VLA4 ( $\alpha 4\beta 1$ ). Cells were observed using a Zeiss Axioskop epifluorescence microscope. Fluores-

cence in each cell was measured (in arbitrary units) using an image processor (see Materials and Methods). Bar graphs to the right of each image show the mean  $\pm$  SEM fluorescence counts. \*Represents a significant difference by unpaired Student's *t*-test analysis with  $P < 0.0001$ .

and FITC-conjugated anti- $\alpha 4\beta 1$  (VLA4) antibody, respectively. As shown in Figure 2 and Table 1, cell surface expression of the transferrin receptor fell by almost two thirds in cells treated with chloramphenicol (10  $\mu\text{g/ml}$ ) for 4 days. The decrease was comparable to that seen in

control cells treated with ferrotransferrin. An extensive body of literature shows that addition of iron-saturated transferrin to cells reduces transferrin receptor cell surface expression, whereas addition of an iron chelator, desferrioxamine, enhances expression. These observa-

TABLE 1. Cell surface expression of the transferrin receptor, CD58, and VLA4<sup>1</sup>

Treatment	Transferrin receptor		CD58		VLA4	
	Fluorescence counts	n <sup>2</sup>	Fluorescence counts	n	Fluorescence counts	n
No treatment	23.6 ± 1.9	70	145.4 ± 10.8	28	13.3 ± 0.7	31
Chloramphenicol	8.6 ± 0.4	104	124.2 ± 8.7	22	12.0 ± 0.9	19
Iron (0.5 μM transferrin)	7.1 ± 0.7	42	Not measured	—	Not measured	—
Desferrioxamine (100 μM)	30.8 ± 1.1	33	Not measured	—	Not measured	—

<sup>1</sup> Fluorescence counts (arbitrary units) are expressed as the mean ± SEM.

<sup>2</sup> n = number of cells counted for fluorescence quantitation.

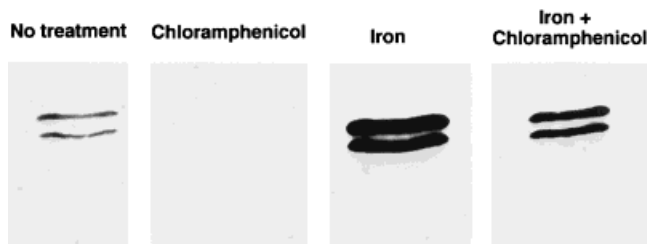


Fig. 3. Ferritin synthesis. K562 cells were treated with chloramphenicol (10 μg/ml) for 4 days, iron (ferric ammonium citrate, 200 μg/ml) for 1 day, or both chloramphenicol (4 days) and iron (for 1 day—3 days after chloramphenicol addition). Cells were then incubated in medium containing <sup>35</sup>S-methionine for 1 h at 37°C and lysed. Ferritin was immunoprecipitated with anti-human ferritin antibody and Protein A sepharose beads. Samples were run on a 15% SDS polyacrylamide gel, followed by fluorography and autoradiography.

tions were confirmed in the present study. In contrast to the transferrin receptor, cell surface expression of CD58 and VLA4 was similar to control values (Table 1).

#### Chloramphenicol decreases ferritin synthesis

Ferritin synthesis was measured by <sup>35</sup>S-methionine metabolic labeling for 1 h followed by SDS-PAGE, fluorography, and autoradiography. As shown in Figure 3, chloramphenicol decreased ferritin synthesis by an average of threefold over three separate experiments compared to untreated controls. Further, cells treated with both chloramphenicol and iron (FeAc, 200 μg/ml, 24 h) did not induce ferritin synthesis to the same degree as cells treated with iron alone.

#### Chloramphenicol decreases protein synthesis

The rate of protein synthesis, measured by total <sup>35</sup>S-methionine incorporation into protein over a 1-h period, was decreased by an average of only 17% over three separate experiments in chloramphenicol-treated cells (data not shown). Thus, generalized suppression of protein synthesis was not the only explanation for the reduced rate of ferritin synthesis.

#### Chloramphenicol decreases transferrin receptor mRNA but not H-ferritin or L-ferritin mRNA

Northern hybridization experiments revealed a 2.5-fold decrease in transferrin receptor message levels in cells treated with chloramphenicol (10 μg/ml) for 4 days (Fig. 4). As expected, control cells treated with iron (FeAc, 200 μg/ml) or desferrioxamine (100 μM), an iron chelator, for 24 h, showed a decrease (fourfold) and

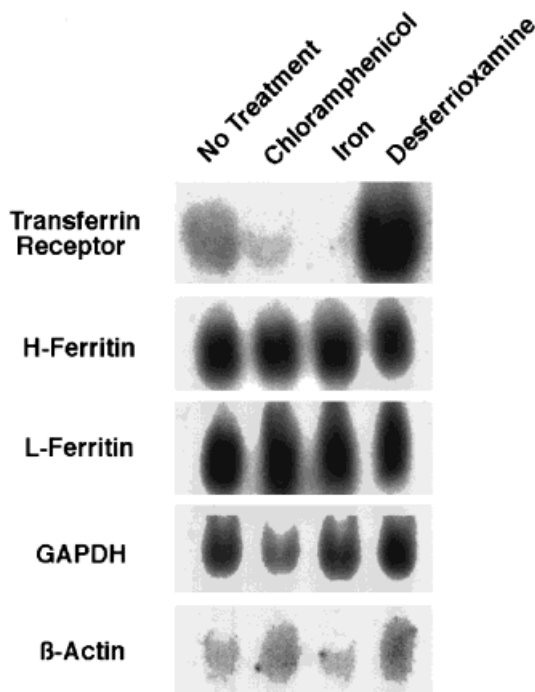


Fig. 4. mRNA levels of the transferrin receptor, H-ferritin, L-ferritin, GAPDH, and β-actin in K562 cells treated with chloramphenicol (10 μg/ml) for 4 days. Cells were also treated with iron (ferric ammonium citrate, 200 μg/ml) or desferrioxamine (100 μM) for 24 h as controls for decreased and increased transferrin receptor message expression, respectively.

an increase (3.7-fold) in message levels, respectively. In contrast, neither H-ferritin nor L-ferritin mRNA levels were perturbed by chloramphenicol. The RNA levels of GAPDH and β-actin were also examined. Interestingly, GAPDH mRNA levels fell twofold in cells treated with chloramphenicol while the β-actin message rose slightly (30%). Desferrioxamine treatment also increased β-actin mRNA levels (40%).

#### Chloramphenicol does not alter IRE-IRP interactions

IRE-IRP interactions in chloramphenicol-treated cells were assessed by a gel retardation assay. As shown in Figure 5, no differences in the amount of protein binding to <sup>32</sup>P-labeled IRE probes were observed between untreated and chloramphenicol-treated cells. As expected, protein binding was enhanced in desferrioxamine-treated



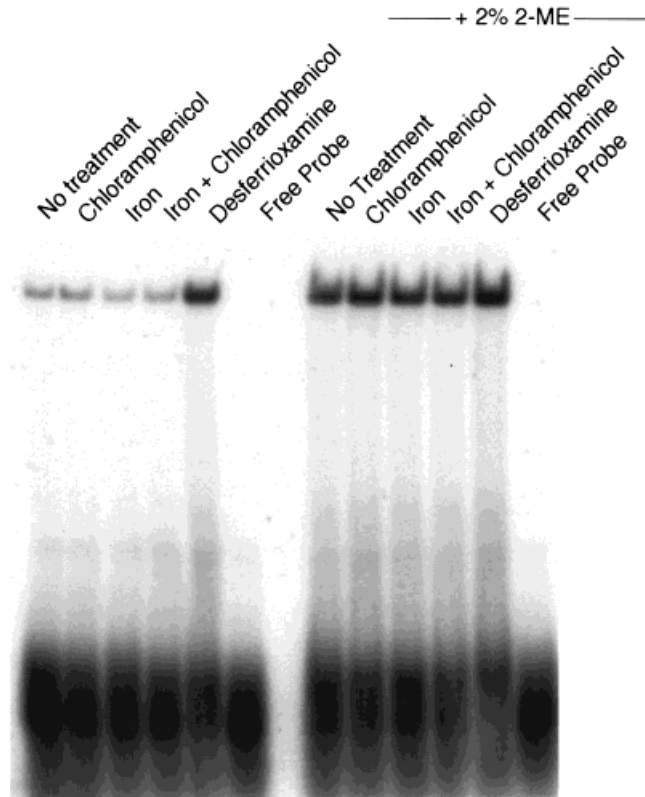


Fig. 5. Gel retardation assay. K562 cells were treated with chloramphenicol (10  $\mu\text{g/ml}$ ) for 4 days, iron (ferric ammonium citrate, 200  $\mu\text{g/ml}$ ) for 1 day, both chloramphenicol (4 days) and iron (1 day), or desferrioxamine (100  $\mu\text{M}$ ) for 1 day. Cell lysates (20  $\mu\text{g}$ ) were incubated with excess  $^{32}\text{P}$ -labeled IRE probe. An additional set of reactions also included 2% 2-mercaptoethanol, which activates IRP and thus allows determination of total cellular IRP. RNA-protein complexes were resolved on a 10% nondenaturing gel.

cells. Full activation of IRP by 2-mercaptoethanol revealed no differences in total cellular IRP between chloramphenicol and untreated cells.

#### Chloramphenicol decreases aconitase activity

Cytosolic aconitase activity was reduced in chloramphenicol-treated cells, as well as in cells treated with both iron and chloramphenicol (Fig. 6). As expected, aconitase activity in desferrioxamine-treated cells was reduced compared to both untreated and iron-treated cells.

### DISCUSSION

The goal of our study is to learn whether chloramphenicol-induced mitochondrial impairment is associated with alterations in some of the major proteins regulating iron metabolism and, if so, to identify the mechanisms underlying those changes. We are specifically interested in erythroid cells since these cells are the highest users of iron in the body and since they are, for unknown reasons, specifically targeted by chloramphenicol.

Chloramphenicol, a powerful antibiotic, consistently causes a dose-dependent suppression of erythropoiesis and occasionally produces sideroblastic changes in the

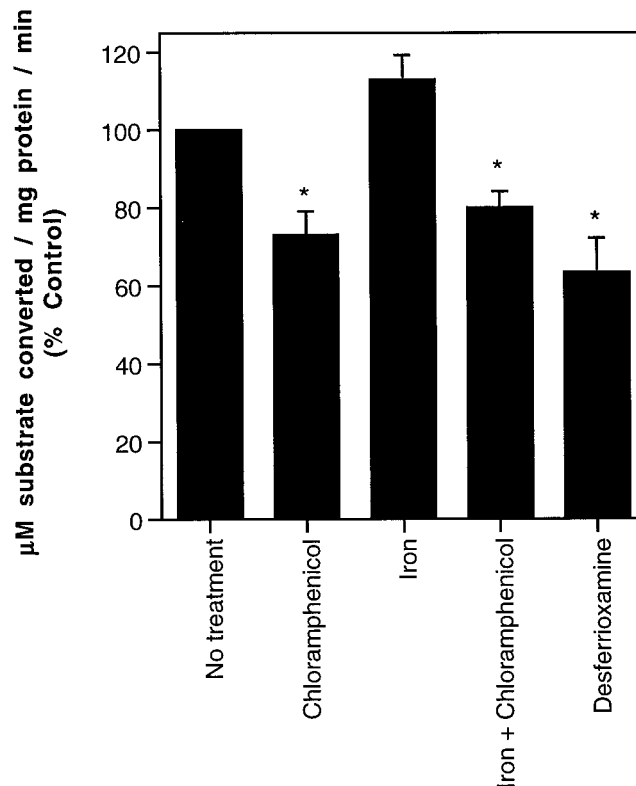


Fig. 6. Cytosolic aconitase activity. K562 cells were treated with chloramphenicol (10  $\mu\text{g/ml}$ ) for 4 days, iron (ferric ammonium citrate, 200  $\mu\text{g/ml}$ ) for 1 day, both chloramphenicol (4 days) and iron (1 day), or desferrioxamine (100  $\mu\text{M}$ ) for 1 day. Cell lysates (50  $\mu\text{g}$ ) were added to cuvettes containing 0.2 mM *cis*-aconitate in a 1-ml assay buffer. Activity was measured spectrophotometrically by the disappearance of *cis*-aconitate at O.D. 240 nm after 10 min. Activity is expressed as a percent of untreated control values (which average approximately 0.15  $\mu\text{M}$  substrate converted/mg protein/min). Values are the mean  $\pm$  SEM,  $n = 8$ . \*Represents significant differences ( $P \leq 0.0001$ ) compared to untreated and iron-treated cells by one-way ANOVA and Fisher's LSD test.

bone marrow. The drug has also been linked to fatal aplastic anemia, which is the primary reason chloramphenicol is no longer routinely prescribed in the United States (Yunis and Salem, 1980). Mitochondrial dysfunction is believed to be the primary cause of the drug's toxic effects. Even in concentrations within therapeutic levels, chloramphenicol, both in vitro and in vivo, reversibly inhibits mitochondrial protein synthesis (Martelo et al., 1969). We confirmed this inhibition in K562 cells in which chloramphenicol also caused a marked reduction in the activity of cytochrome *c* oxidase, the terminal enzyme in respiration, as well as total cellular ATP levels, which were 11% and 45% of control levels, respectively (Fig. 1B,C). These decreases were not unexpected, since chloramphenicol inhibits the synthesis of three subunits of cytochrome *c* oxidase and two subunits of ATPase, all of which are encoded on the mitochondrial genome (Attardi, 1993). Consistent with this, we found a statistically significant 20% reduction in respiratory activity (Fig. 1D). Respiration was measured by incubating cells with a mitochondria-specific probe that fluoresces in proportion to the amount of oxygen entering the cell. It is noteworthy



that an even lower activity may be masked by the likely presence of reactive oxygen species in chloramphenicol-treated cells, since these molecules can also cause the probe to fluoresce. In agreement with our observations, previous studies have found decreased oxygen uptake in human leukocytes (Follette et al., 1956) and in the basal metabolic rate in rats (Hallman, 1973) after chloramphenicol administration.

Inhibition of the synthesis of mitochondrial-DNA-coded respiratory chain enzymes and, hence, energy-dependent processes, is thought to be responsible for the cessation of cellular proliferation in a variety of cells, including HeLa (Firkin and Linnane, 1968) and bone marrow (Ratzan et al., 1974). In our study, K562 cell growth progressively declined over a 1-week period after treatment with a range of chloramphenicol concentrations found in patients' plasma (10, 25, and 40  $\mu\text{g/ml}$ ) (Fig. 1A) (Drug Information for the Health Care Professional, 1995). Cell growth inhibition was associated with a gradual increase in the percent of cells in the G1 phase of the cell cycle and a gradual decrease in the percent of cells in S-phase (data not shown). It is possible that the accumulation of cells in G1 is the manifestation of cellular signals sensing ATP reductions (Dorward et al., 1997).

Chloramphenicol decreased cell surface transferrin receptor expression (Fig. 2). Iron uptake is mediated exclusively by transferrin in developing red cells and is essential for heme synthesis and for the proliferation and maturation of these cells (Ponka, 1997). Thus, a decrease in the number of transferrin receptors could decrease iron uptake and thereby play a role in the erythropoietic abnormalities and in the hypochromic-microcytic anemia observed in patients susceptible to chloramphenicol toxicity (Bottomley, 1982). Also, this observation is in keeping with findings of increased plasma iron and decreased plasma  $^{59}\text{Fe}$  clearance in these individuals (Yunis and Salem, 1980). The decrease in cell surface transferrin receptor expression was accompanied by a 2.5-fold reduction in mRNA levels (Fig. 4). It is not clear from our studies whether the decrease in transferrin receptor message levels after chloramphenicol treatment is due to decreased transcription or enhanced message degradation.

Chloramphenicol also caused a decrease in de novo ferritin synthesis in K562 cells (Fig. 2) which was not accompanied by a decrease in mRNA levels (Fig. 4). Ferritin synthesis was appropriately induced by ferric ammonium citrate (iron) after chloramphenicol treatment, but not to the same extent as in cells treated with iron alone (Fig. 3). This finding may indicate that ferritin synthesis is reduced in chloramphenicol-treated cells by a mechanism that is in part independent of the mechanism by which transferrin receptor expression is decreased, since  $\text{Fe}^{3+}$  can permeate K562 cells by a pathway independent of transferrin (Inman et al., 1994; Inman and Wessling-Resnick, 1993). However, it is also possible that the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  by K562 cell surface ferrireductase is impaired, since this reaction is presumably a requirement for nontransferrin iron transport (Inman et al., 1994).

Since transferrin receptor expression was reduced, the finding of decreased ferritin synthesis was interesting. Numerous studies in nonerythroid cells have shown that ferritin and the transferrin receptor are

regulated inversely at the translational level by the IRP, which actually exists in two forms (IRP-1 and IRP-2). In these cells, binding of the IRP to IREs on transferrin receptor mRNA stabilizes this message, whereas binding of this protein to an IRE on ferritin mRNA inhibits its translation on ribosomes. Thus, when expression of one is high, expression of the other is low (Harford et al., 1994). The concomitant decrease in both the transferrin receptor and ferritin in chloramphenicol-treated K562 cells in our study implies that, in certain circumstances, a different mode of regulation may be in effect. This is not actually surprising since there is much evidence for unique modes of regulation of iron metabolism in erythroid cells (Ponka, 1997). Indeed, many studies in such cells would appear to contradict the IRE/IRP control theory, demonstrating, for example, unidirectional increases in both transferrin receptor expression and erythroid aminolevulinic acid synthase (ALA-S; Ponka 1997). ALA-S, the first enzyme in the heme biosynthetic pathway, contains an IRE in its 5' UTR (untranslated region) and has been shown to be translationally regulated by the IRP in a manner analogous to ferritin (Dierks, 1990; Cox et al., 1991; Dandekar et al., 1991). In our study, the binding affinity of the IRP to the IRE in chloramphenicol-treated cells was unchanged compared to untreated cells (Fig. 5). We cannot say with certainty that this was true for both IRP-1 and IRP-2, since the two proteins cannot be resolved with gel retardation assays in human cells. Interestingly, chloramphenicol-treated cells also displayed decreased cytosolic aconitase activity (Fig. 6). Purified IRP-1 has been shown to have aconitase activity and may provide much of the cytoplasmic aconitase activity in cells (Hentze and Argos, 1991; Kaptain et al., 1991; Rouault et al., 1991). Aconitase catalyzes the interconversion of citrate and isocitrate via *cis*-aconitate (Zheng et al., 1992). Many studies have demonstrated that IRE binding and aconitase activities of the IRP are inversely coupled depending on the level of iron in the cell (Ponka, 1997). Thus, with low intracellular iron, IRP-1 contains a [3Fe-4S] cluster and has high IRE binding affinity and low aconitase activity, whereas in iron-replete cells, IRP-1 contains a [4Fe-4S] cluster with low IRE binding and high aconitase activities. In our study, the fact that chloramphenicol-treated cells had low cytosolic aconitase activity without increased IRP/IRE binding—even when iron was also added to these cells (Fig. 6)—suggests that signals from the mitochondria may override iron regulation of cytosolic aconitase activity. Possibly, this enzyme, in certain circumstances, is regulated similarly to the more well-known mitochondrial aconitase in the Krebs cycle. It seems possible that this enzyme would be inhibited by rising levels of Krebs cycle intermediates. An elevation in intermediates upstream of the electron transport chain may also explain the reduction in message levels of GAPDH (Fig. 4), an important enzyme in the glycolysis pathway (Lehninger, 1982).

The association of mitochondrial dysfunction with decreased synthesis of the transferrin receptor and ferritin is interesting. One may speculate that the changes are due to a general decline in the cell's synthetic machinery as a result of ATP depletion. Indeed, total protein synthesis was moderately reduced (17%) in chloramphenicol-treated cells (data not shown). Yet,

the process is obviously selective, since no decreases were observed in cell surface expression of the cellular adhesion molecules CD58 or VLA4 (Fig. 2). In addition, ferritin mRNA levels were not decreased and  $\beta$ -actin mRNA levels were slightly increased in chloramphenicol- as well as in desferrioxamine-treated cells (Fig. 4). This last result is intriguing, since recent studies suggest that  $\beta$ -actin plays a role in cell growth (Mashima et al., 1997) and/or apoptosis (Villa et al., 1988; Brown et al., 1997); and reduced growth and induction of apoptosis have been associated with both treatments (Holt et al., 1997; Hoffbrand et al., 1976; Lederman et al., 1984; Riaz-Ul-Haq et al., 1995).

Alternatively, one may speculate that downregulation of transferrin receptors is a deliberate attempt by the cell to conserve energy by reducing growth while sparing the cell of the energy-expensive process of transferrin-mediated iron uptake and delivery (Ponka, 1997). Yet, a reduction in ATP levels alone would appear not at fault. Previous studies in our laboratory in K562 cells showed that while cellular ATP depletion decreases the translational mobility of cell surface transferrin receptors, it actually increases the number of transferrin receptors on the cell surface (Thatte et al., 1996). Another possibility is that defective heme synthesis, due to mitochondrial impairment, decreases transferrin receptor expression. Indeed, others have shown that inhibition of heme synthesis depresses transferrin receptor synthesis in reticulocytes (Cox et al., 1985) and erythroid cells (Grdisa et al., 1993). However, previous studies in our laboratory found that complete inhibition of heme synthesis by succinylacetone had no effect on iron uptake in K562 cells (Bottomley et al., 1985). A third possible explanation is that reduced transferrin receptor expression occurs secondary to the observed decrease in ferritin synthesis (Fig. 3). Decreased ferritin synthesis may expand the chelatable iron pool and thus downregulate transferrin receptor expression, since it is thought that the size of this intracellular transit iron pool reflects available iron stores within the cell and hence regulates iron acquisition (Halliday et al., 1994).

The relationship of ferritin synthesis to mitochondrial dysfunction is unclear. However, it is tempting to speculate that ferritin synthesis is decreased in conjunction with iron release from ferritin. One factor causing iron release may be enhanced oxygen radical production. Indeed, it is well established that free radicals cause iron release from ferritin (Halliday et al., 1994). Also, it has been postulated that defects in respiratory enzymes reduce the availability of ferrous iron for ferrochelatase, the last enzyme in the heme synthetic pathway, and lead to accumulation of ferric iron in mitochondria (Ponka, 1997). Hence, iron release and decreased ferritin synthesis may be a compensatory response under these circumstances in an effort to deliver ferrous iron to mitochondria. Also, there is much interest today in the links between iron and citrate metabolism (Harrison and Arosio, 1996). One may speculate that elevated citrate levels under these circumstances may influence iron release from ferritin (Treffry and Harrison, 1979).

In summary, we have shown that chloramphenicol-induced mitochondrial inhibition is associated with marked decreases in transferrin receptor expression

and ferritin synthesis in K562 cells. The changes mediated by chloramphenicol are distinct from the well-characterized regulation of ferritin and transferrin receptor regulation by the IRP/IRE interaction. These data imply an association between mitochondrial function and specific regulation of nonmitochondrial proteins and may also provide clues about the pathways by which hematotoxicities develop. Red blood cell precursors have a high demand for iron for heme synthesis, and, quantitatively, most of the iron in the body is found within the erythron (Hillman and Finch, 1992). Normoblasts differ from most other cells in their nearly complete dependence on transferrin receptors for iron uptake (Ponka, 1997). Thus, developing normoblasts are likely to be more susceptible to chloramphenicol's disturbances in iron homeostasis. Perhaps certain individuals have preexisting abnormalities in mitochondrial function or iron homeostasis, which make them more vulnerable to the drug's effects.

Since chloramphenicol continues to be used worldwide, particularly in developing countries, it is important to understand how this drug causes bone marrow toxicities. Future studies in our laboratory will investigate the mechanisms by which mitochondrial dysfunction alters iron metabolism. It will be important to ascertain whether the effect is selective and whether it is a general occurrence in many cell types.

#### ACKNOWLEDGMENTS

We thank Dr. Jack Rogers for providing the transferrin receptor and the H- and L-ferritin cDNA for Northern hybridization probes. This work was supported by an NIH grant (R01-HL45794-07) (K.R. Bridges and L.M. Leiter). H.S. Thatte was supported by an NIH National Research Service Award and by NIH grant (R01HL-15157 and R01HL-32854) (D.E. Golan). Peter W. Marks was supported by an NHLBI grant (K08-HL-03235).

#### LITERATURE CITED

- Attardi G. 1993. The human mitochondrial genetic system. In: DiMauro S, Wallace DC, editors. Mitochondrial DNA in human pathology. New York: Raven Press. p 9-25.
- Bartz QR. 1948. Isolation and characterization of chloromycetin. *J Biol Chem* 172:445-450.
- Bottomley SS. 1982. Sideroblastic anemia. *Clin Haematol* 11:389-409.
- Bottomley SS, Wolfe LC, Bridges KR. 1985. Iron metabolism in K562 erythroleukemic cells. *J Biol Chem* 260:6811-6815.
- Brown SB, Bailey K, Savill J. 1997. Actin is cleaved during constitutive apoptosis. *Biochem J* 323:233-237.
- Constanzo T, Colombo M, Staempfli S, Marone G, Frank R, Delius H, Cortese R. 1986. Structure of gene and pseudogenes of human apoferritin H. *Nucleic Acids Res* 14:721-736.
- Cox TC, Bawden MJ, Martin A, May BK. 1991. Human erythroid  $\delta$ -aminolevulinic acid synthase: promoter analysis and identification of an iron-responsive element in the mRNA. *EMBO J* 10:1891-1902.
- Cox TM, O'Donnel MW, Aisen P, London IM. 1985. Biosynthesis of the transferrin receptor in rabbit reticulocytes. *J Clin Invest* 76:2144-2150.
- Dandekar T, Stripecke R, Gray NK, Goossen B, Constable A, Johansson HE, Hand Hentz MW. 1991. Identification of a novel iron-responsive element in murine and human erythroid  $\delta$ -aminolevulinic acid synthase mRNA. *EMBO J* 10:1903-1909.
- Davies JR, Scopes RK. 1981. Purification of some tricarboxylic acid cycle enzymes from beef heart using affinity elution chromatography. *Anal Biochem* 114:19-27.
- Dente L, Ciliberto G, Cortese R. 1985. Structure of the human alpha 1-acid glycoprotein gene: sequence homology with other human acute phase protein genes. *Nucleic Acids Res* 13:3941-3952.
- Dierks P. 1990. Molecular biology of eukaryotic  $\delta$ -aminolevulinic acid

- synthase. In: Dailey HA, editor. Biosynthesis of heme and chlorophylls. New York: McGraw-Hill. p 201.
- Dorward A, Sweet S, Moorehead R, Singh G. 1997. Mitochondrial contributions to cancer cell physiology: redox balance, cell cycle, and drug resistance. *J Bioenerg Biomembr* 29:385-392.
- Drug Information for the Health Care Professional. 1995. USPDI (15th ed.). Taunton, MA: Rand McNally. p 716-724.
- Ehrlich J, Bartz QR, Smith RM, Joslyn DA, Burkholder PR. 1947. Chloromycetin, a new antibiotic from a soil actinomycete. *Science* 106:417.
- Firkin FC, Linnane AW. 1968. Differential effects of chloramphenicol on the growth and respiration of mammalian cells. *Biochem Biophys Res Commun* 32:398-402.
- Follette JH, Shugarman PM, Reynold J, Valentine WN, Lawrence JS. 1956. The effect of chloramphenicol and other antibiotics on leukocyte respiration. *Blood* 11:234-242.
- Grdisa M, Mathew A, Johnstone RM. 1993. Expression and loss of the transferrin receptor in growing and differentiating HD3 cells. *J Cell Physiol* 155:349-357.
- Halliday JW, Ramm GA, Powell LW. 1994. Cellular iron processing and storage: the role of ferritin. In: Brock JH, Halliday JW, Pippard MJ, Powell LW, editors. Iron metabolism in health and disease. Philadelphia: Saunders. p 97-121.
- Hallman M. 1973. Oxygen uptake in neonatal rats: a developmental study with particular reference to the effects of chloramphenicol. *Pediatr Res* 7:923-930.
- Harford JB, Rouault TA, Klausner RD. 1994. The control of cellular iron homeostasis. In: Brock JH, Halliday JW, Pippard MJ, Powell LW, editors. Iron metabolism in health and disease. Philadelphia: Saunders. p 123-149.
- Harrison PM, Arosio P. 1996. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta* 1275:161-203.
- Hayes DJ. 1994. Experimentally induced defects in mitochondrial function. In: Darley-Usmar V, Schapira AHV, editors. Mitochondria: DNA, proteins and disease. Chapel Hill, NC: Portland Press. p 157-178.
- Hentze MW, Argos P. 1991. Homology between IRE-BP, a regulatory RNA-binding protein, aconitase, and isopropylmalate isomerase. *Nucleic Acids Res* 19:1739-1740.
- Higuchi Y, Linn S. 1995. Purification of all forms of HeLa cell mitochondrial DNA and assessment of damage to it caused by hydrogen peroxide treatment of mitochondria or cells. *J Biol Chem* 270:7950-7956.
- Hillman RS, Finch CA. 1992. Red cell manual (6th ed.). Philadelphia: F.A. Davis. p 65-114.
- Hoffbrand AV, Ganeshguru K, Hooton JWL, Tattersall MHN. 1976. Effect of iron deficiency and desferrioxamine on DNA synthesis in human cells. *Br J Haematol* 33:517-526.
- Holt DE, Ryder TA, Fairbairn A, Hurley R, Harvey D. 1997. The myelotoxicity of chloramphenicol: in vitro and in vivo studies. I. In vitro effects on cells in culture. *Hum Exp Toxicol* 16:570-576.
- Inman RS, Wessling-Resnick M. 1993. Characterization of transferrin-independent iron transport in K562 cells. Unique properties provide evidence for multiple pathways of iron transport. *J Biol Chem* 268:8521-8528.
- Inman RS, Coughlan MM, Wessling-Resnick M. 1994. Extracellular ferrireductase activity of K562 cells is coupled to transferrin-independent iron transport. *Biochemistry* 33:11850-11857.
- Kaptain S, Downey WE, Tang C, Philpott C, Haile D, Orloff DG, Harford JB, Rouault TA, Klausner RD. 1991. A regulated RNA binding protein also possesses aconitase activity. *Proc Natl Acad Sci USA* 88:10109-10113.
- Lederman HM, Cohen A, Lee JWW, Freedman MH, Gelfand EW. 1984. Deferoxamine: a reversible S-phase inhibitor of human lymphocyte proliferation. *Blood* 64:748-753.
- Lehninger AL. 1982. Electron transport, oxidative phosphorylation, and regulation of ATP production. In: Anderson S, Fox J, editors. Principles of biochemistry. New York: Worth Publishers. p 467-510.
- Leibold EA, Munro HN. 1988. Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs. *Proc Natl Acad Sci USA* 85:2171-2175.
- Martelo OJ, Manyan DR, Smith US, Yunis AA. 1969. Chloramphenicol and bone marrow mitochondria. *J Lab Clin Med* 74:927-940.
- Mashima T, Mikiyoko N, Noguchi K, Miller DK, Nicholson DW. 1997. Actin cleavage by CPP-32/apopain during the development of apoptosis. *Oncogene* 14:1007-1012.
- McClelland A, Kühn LC, Ruddle FH. 1984. The human transferrin receptor gene: genomic organization, and the complete primary structure of the receptor deduced from a cDNA sequence. *Cell* 39:267-274.
- Mullner EW, Neupert B, Kuhn LC. 1989. A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. *Cell* 58:373-382.
- Ponka P. 1997. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. *Blood* 89:1-25.
- Ratzen RJ, Moore MAS, Yunis AA. 1974. Effect of chloramphenicol on the in vitro colony-forming cell. *Blood* 43:363-369.
- Riaz-ul-Haq, Wereley JP, Chitambar CR. 1995. Induction of apoptosis by iron deprivation in human leukemic CCRF-CEM cells. *Exp Hematol* 23:428-432.
- Rouault TA, Stout CD, Kaptain S, Harford JB, Klausner RD. 1991. Structural relationship between an iron-regulated RNA-binding protein (IRE-BP) and aconitase: functional implications. *Cell* 64:881-883.
- Smith L. 1955. Spectrophotometric assay of cytochrome c oxidase. *Methods Biochem Anal* 2:427-433.
- Thattai HS, Bridges KR, Golan DE. 1996. ATP depletion causes translational immobilization of cell surface transferrin receptors in K562 cells. *J Cell Physiol* 166:446-452.
- Treffry A, Harrison PM. 1979. The binding of ferric iron by ferritin. *Biochem J* 181:709-716.
- Villa P, Henzel W, Sensenbrenner M, Henderson C, Pettman B. 1988. Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis. *J Cell Sci* 111:713-722.
- Yunis AA, Salem Z. 1980. Drug-induced mitochondrial damage and sideroblastic change. *Clin Haematol* 9:607-619.
- Zheng L, Kennedy MC, Beinert H, Zalkin H. 1992. Mutational analysis of active site residues in pig heart aconitase. *J Biol Chem* 267:7895-7903.