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PRINCIPAL INVESTIGATOR: John D. Kemp, M.D.

CONTRACTING ORGANIZATION: University of Iowa
Iowa City, Iowa 52242

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FOREWORD

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Introduction

The problem being addressed is the need for improved therapy of metastatic breast cancer. There are now clear indications that iron deprivation treatment is useful clinically against several tumor types. We have now published evidence that iron deprivation inhibits breast cancer cell lines in vitro and that iron deprivation can be employed in vivo. We are continuing to analyze the mechanisms underlying the anti-tumor effects of iron deprivation treatment and its potential utility in pre-clinical experimental systems involving breast cancer.

Body

During the 1996 calendar year, progress has been made towards completing the work described for all five Specific Aims (Task Statements). Perhaps the most interesting findings have developed in relation to Aim #4, as will be described below. We will, however, approach each of the Aims in numerical sequence.

In relation to Specific Aim #1 (Task #1), it can be recalled from last year's report that we showed that monoclonal antibodies against the transferrin receptor produced significant left-shifts in the DFO dose/response curves for two breast cancer cell lines. The findings supported the view that this reagent combination may show useful activity against breast cancer cell lines and were published (1).

We also wanted to work with the MCF-7 cell line in relation to Specific Aim #1, however. Although we initially had some difficulty in obtaining stable growth with the cell line, we were able to stabilize it. We then established the DFO dose/response curve for MCF-7 and a representative set of data are shown in Figure 1 (all Figures and Tables are in the Appendix). It can be seen that half-maximal inhibition of growth (as assessed by the MTT assay) is achieved at about a 7 micromolar concentration of deferoxamine.

At this point we considered whether it might be more productive and informative in the short run to proceed with the DFO/Tamoxifen 2-way dose/response curves (see Specific Aim #5) before proceeding with the DFO/IgG

ATRA experiments. We elected to pursue the former course and therefore established the Tamoxifen dose/response curve for MCF-7. Representative data are shown in Figure 2. It can be seen that half-maximal inhibition (in the MTT assay) occurs at approximately a 10 micromolar dose.

With these data in hand we performed DFO/Tamoxifen 2-way dose/response experiments. In order to simplify the presentation and interpretation of the multiple curves that can be generated from such experiments, key representative data are shown in Table 1. Thus, for a single dose of DFO that is approximately half-maximally inhibitory, the interactions with multiple doses of Tamoxifen are shown in the "observed inhibition" column. For comparison, the effects that might arise from an addition of effects (as could be seen with agents acting in a similar manner) as well as the effects that might arise from multiplication (as could be seen with non-interacting agents acting in independent manners) are also shown.

It is clear that the observed inhibition is, for all doses, less than that expected from either the similar effect or independent effect concepts. Similar data were obtained for other doses of DFO and for cultures carried out for 120 hours (Table 2). This pattern of findings suggests that the DFO/Tamoxifen interaction is subadditive or antagonistic. This kind of result might be explained in the following manner: since DFO is, at limiting doses, most likely to be S-phase specific (by virtue of inhibiting ribonucleotide reductase), its effect might be impeded by a reduction in the stimulus for cell cycle entry caused by Tamoxifen exposure. These experiments are not yet complete and further data will be collected during 1997.

In relation to the pursuit of Specific Aim #2, recent publications by other groups have steadily strengthened the view that the inhibitory effects of deferoxamine can be specifically and stoichiometrically reversed by iron in various experimental systems (2-4). While it remains possible that similar observations might not occur with breast cancer cell lines, it seems increasingly less likely. Partly for this reason, we have temporarily lowered the priority level of the experiments

described for Task 2 in the application. We will, however, complete the work during the course of the support provided.

A very interesting related point has arisen in recent work, however, and we were able to capitalize on it by virtue of our continuing collaboration with a scientist from the Czech republic, Dr. Jan Kovar (who was supported by the grant 1995 and who subsequently returned to Prague). The point of interest is that iron deprivation, like several other forms of cancer treatment, induces programmed cell death, or apoptosis (2-4). Through our collaboration with Dr. Kovar we were able to provide the most compelling, most direct evidence yet available to support the view that iron deprivation specifically induces apoptosis. A manuscript pertaining to this work has been submitted for publication and a copy is attached to this report. Although Dr. Kovar chose not to direct this work specifically at a breast cancer cell line, it is clear that his earlier work (which was supported by the Breast Cancer Grant) set the stage for the apoptosis experiments. Thus, the Breast Cancer Grant is acknowledged in the paper that has been submitted. It is now clearly of interest to ask whether iron deprivation produces apoptosis in breast cancer cell lines and we are currently contemplating the design of such experiments. The development of this new avenue of investigation is thus an unanticipated benefit of the work supported by the Army Breast Cancer Grant.

In relation to the pursuit of Specific Aim #3 we had previously ascertained (as noted in our Annual Report for 1995) that the MDA-MB-231 cell line will be our initial test target for combined treatment in vivo with HES-DFO and monoclonal antibodies against the transferrin receptor. We had anticipated that we would be able to begin the experiments with an adequate supply of monoclonal antibodies A27 and E2.3. We experienced difficulty in obtaining adequate yields of the antibodies, however, and consulted with Dr. Ray Taetle (University of Arizona) and Dr. Toby Hecht (National Cancer Institute) who have also been producing these same antibodies by the same method (ascites in mice) for a Phase I/II Clinical Trial

involving lymphoma patients that is expected to commence in 1997. By following their advice, we were able to increase our production of the antibodies to some extent. We now have 340 milligrams of E2.3 stored and 385 milligrams of A27.15 stored. This is now enough to begin experimentation in vivo while we continue to make more antibodies .

In an attempt to make maximally effective use of our resources while dealing with the delay in relation to Specific Aim #3, we elected to, a) intensify our efforts in relation to Specific Aim #4 (to be discussed below) and to, b) investigate the possibility that DFO might interact with other chemotherapeutic agents are known to be active against breast cancer. In particular, we chose to investigate doxorubicin. We therefore established the doxorubicin dose/response curves for the SKBR-3, MDA-MB-231, and MCF-7 cell lines and these data are shown in Figures 3,4, and 5. We then undertook two way dose/response analyses similar to those described above for DFO and Tamoxifen. Key data are shown in Tables 3, 4, and 5 which are constructed in a format similar to Tables 1 and 2.

The findings indicate that for one of the three cell lines, SKBR-3, combined treatment produces growth inhibition at a level which is between the multiplicative and additive predictions while for the other two cell lines (MDA-MB-231 and MCF-7) combined treatment produces inhibition which is below both the additive and multiplicative predictions. These results raise the possibility that at least some breast cancers may respond favorably to combined DFO/Doxorubicin treatment but also beg the question as to why only some tumors are sensitive. We currently plan to conduct additional experiments of this type and publish them in conjunction with the results of the DFO/Tamoxifen experiments described above.

In relation to the pursuit of Specific Aim #4 (which pertains to the analysis of toxicity of iron deprivation treatment), we have made what we consider to be a very important new observation. We undertook a new exploration partly because we were

hindered in relation to Aim # 3 and partly because we felt that we had developed a significant new idea.

The new observation may provide an important new insight into the most severe and puzzling systemic toxicity that occurs in mice after approximately six days of continuous treatment in vivo with both HES-DFO and a pair of monoclonal antibodies against the transferrin receptor (the toxicity is not seen with either HES-DFO alone or monoclonal antibodies alone). The mice exhibit signs of stress (hypomotility, piloerection) and will go on to die in a few days in almost all cases. Although earlier work with a single antibody/HES-DFO protocol led us to suspect that they might be at risk for infection, autopsy studies showed neither infection nor hemorrhages (5). The findings were puzzling because infection and hemorrhage are the two most likely complications of bone marrow suppression and we had assumed that the bone marrow would be the most likely target of toxicity.

After reviewing a recent paper by Weiss et al. (6) (which shows that iron depletion in murine macrophages can enhance macrophage activation and increase production of nitric oxide by virtue of increasing transcription of the nitric oxide synthase gene), we decided to test for nitric oxide (NO) production in our mice by means of electron paramagnetic resonance (EPR) analysis of whole blood samples. The data presented in Figure 6 and the legend for Figure 6 illustrate and summarize the key findings. A third of the treated mice exhibited elevated NO levels. All of the mice, however, exhibited elevated ceruloplasmin levels. The latter is an acute phase reactant protein that falls within the subset that is most likely to be induced by IL-6 (7). Although more work needs to be done, these early studies are nevertheless consistent with the hypothesis that there is enhanced macrophage activation (leading to both a significant acute phase response and to an increased propensity for the production of significant levels of nitric oxide) in the acutely iron deprived mice. We consider these findings to be of considerable interest since they may not only help explain the observed toxicity, but also point toward ways in which iron

deprivation toxicity can be reduced. We will therefore consider submitting additional grant applications to provide support for the pursuit of these studies.

Our progress in relation to Specific Aim #5 has already been covered above in the discussion of Specific Aim #1.

Conclusions

Progress has been made in the study of iron deprivation as a potential treatment modality for breast cancer. Enhanced macrophage activation with an augmented acute phase response has been implicated as a source of potential treatment related toxicity. Deferoxamine appears to interact successfully with Doxorubicin in vitro in inhibiting the growth of some breast cancer cell lines. New, direct evidence for iron-deprivation inducing apoptosis arose in a collaborative study and has been submitted for publication. Sufficient monoclonal antibody reagents are now on hand to begin in vivo anti-tumor experiments with the MDA-MB-231 breast cancer cell line.

Progress to date indicates that no insurmountable barriers have been encountered and that all of the proposed research can be accomplished within the time frame originally proposed.

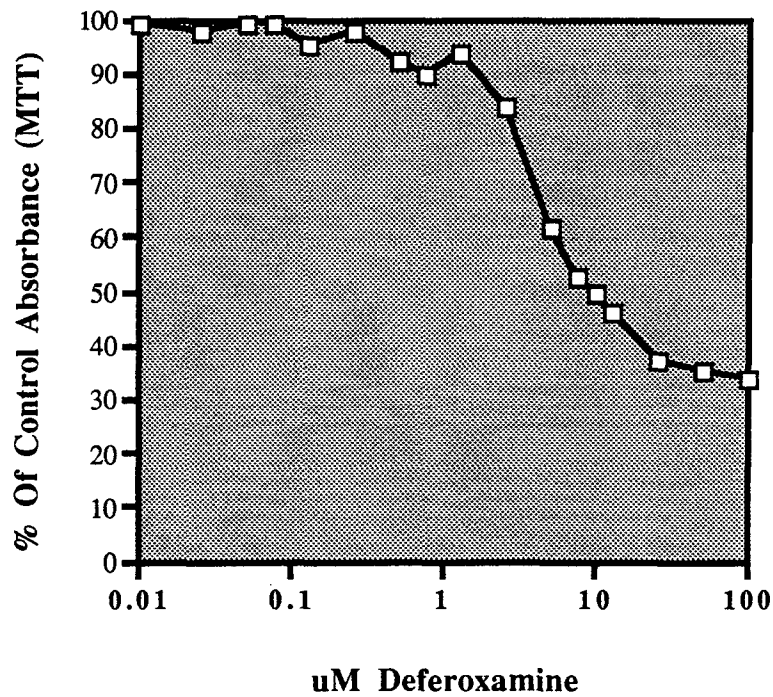
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Figure 1

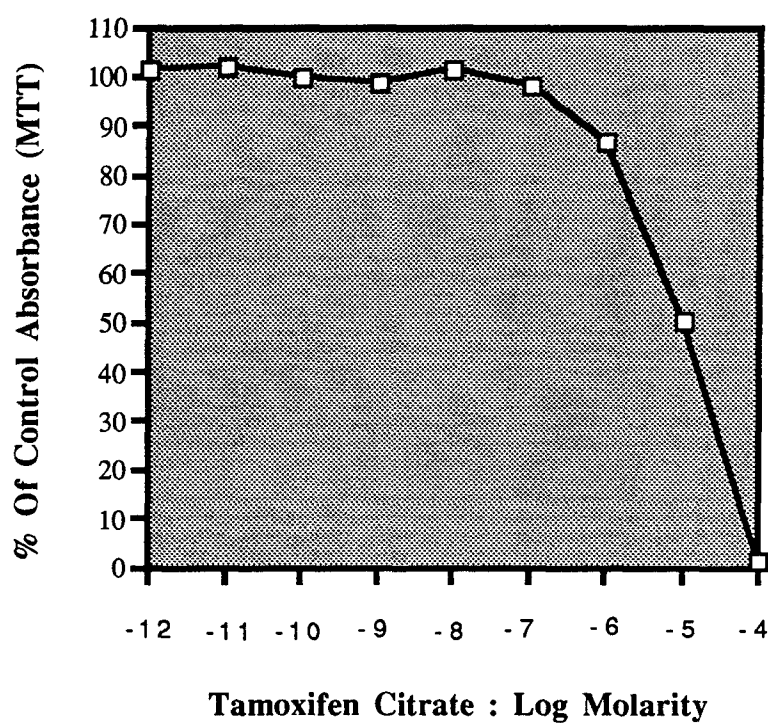
**MCF-7 DFO Dose Response
72 Hour Exposure**



5,000 cells per well were plated in a .1 cc volume in 96 well plates 24 hours prior to the addition of the indicated concentrations of Deferoxamine. Plates were incubated an additional 72 hours prior to the MTT assay. RPMI 1640 was the basic tissue culture media used, with standard supplements.

Figure 2

**MCF-7 Tamoxifen Dose Response
72 Hour Exposure**



5,000 cells per well were plated in a .1 cc volume in 96 well plates 24 hours prior to the addition of the indicated concentrations of Tamoxifen. Plates were incubated an additional 72 hours prior to the MTT assay. RPMI 1640 was the basic tissue culture media used, with standard supplements.

Table 1

Dose of Tamoxifen	Dose of DFO	PIE-A ¹	PIE-M ²	Observed Inhibition
		%	%	%
1x10 ⁻⁷ M	7x10 ⁻⁶ M	61.0	56.1	50.4
5x10 ⁻⁷ M	7x10 ⁻⁶ M	69.1	60.0	51.2
1x10 ⁻⁶ M	7x10 ⁻⁶ M	75.2	62.9	49.7
5x10 ⁻⁶ M	7x10 ⁻⁶ M	81.2	65.9	48.2
1x10 ⁻⁵ M	7x10 ⁻⁶ M	89.2	69.8	56.3
5x10 ⁻⁵ M	7x10 ⁻⁶ M	100.00	100.00	100.0

1. PIE-A = Predicted inhibitory effect by addition of effects.
2. PIE-M = Predicted inhibitory effect by multiplication of effects.

Inhibitory effect of combined treatment with a half-maximal dose of deferoxamine and multiple doses of Tamoxifen (72 hour treatment).

5x10⁴ cells were cultured in 96 well plates in 100 µl of RPMI 1640 based tissue culture media for 24 hours prior to the addition of the test compounds in an additional 100 µl of media. Cultures were then continued for an additional 72 hours prior to the MTT assay. The inhibitory effect of 7x10⁻⁶ DFO was 51.5% while the inhibitory effects of the various doses of Tamoxifen were 9.5%, 17.6%, 23.7%, 29.7%, 37.7%, and 100% for 1x10⁻⁷M, 5x10⁻⁷M, 1x10⁻⁶M, 5x10⁻⁶M, 1x10⁻⁵M, and 5x10⁻⁵M, respectively. Equivalent observations were made at other doses of DFO in this and other experiments. The inhibitory effect of the alcohol vehicle control at the maximum concentration employed (0.5% for 5x10⁻⁵M Tamoxifen) was 8.3%.

Table 2

Dose of Tamoxifen	Dose of DFO	PIE-A ¹	PIE-M ²	Observed Inhibition
		%	%	%
1x10 ⁻⁷ M	4x10 ⁻⁶ M	53.1	52.2	59.9
5x10 ⁻⁷ M	4x10 ⁻⁶ M	71.5	61.1	61.1
1x10 ⁻⁶ M	4x10 ⁻⁶ M	73.9	62.3	59.9
5x10 ⁻⁶ M	4x10 ⁻⁶ M	88.7	69.5	60.0
1x10 ⁻⁵ M	4x10 ⁻⁶ M	100.0	77.5	70.2
5x10 ⁻⁵ M	4x10 ⁻⁶ M	100.00	100.00	100.0

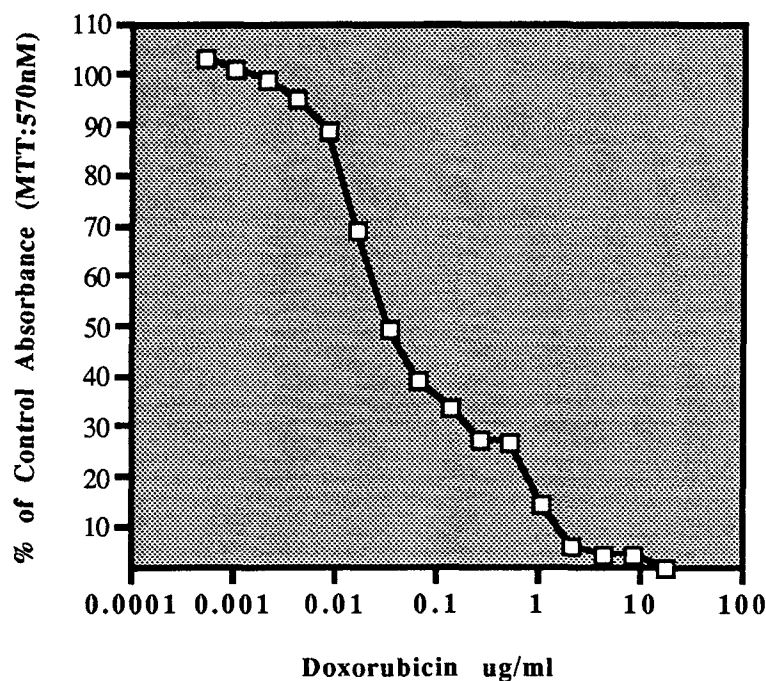
1. PIE-A = Predicted inhibitory effect by addition of effects.
2. PIE-M = Predicted inhibitory effect by multiplication of effects.

Inhibitory effect of combined treatment with a half-maximal dose of deferoxamine and multiple doses of Tamoxifen (120 hour treatment).

The experiment was performed in a manner identical to that described for Table 1 except that the treatment exposure was for 120 hours. The inhibitory effect of DFO at the stated dose was 51.3% while the effects of the doses of Tamoxifen were 1.8%, 20.2%, 22.6%, 37.4%, 53.7%, and 100% inhibition, in order, from the smallest to the largest dose.

Figure 3

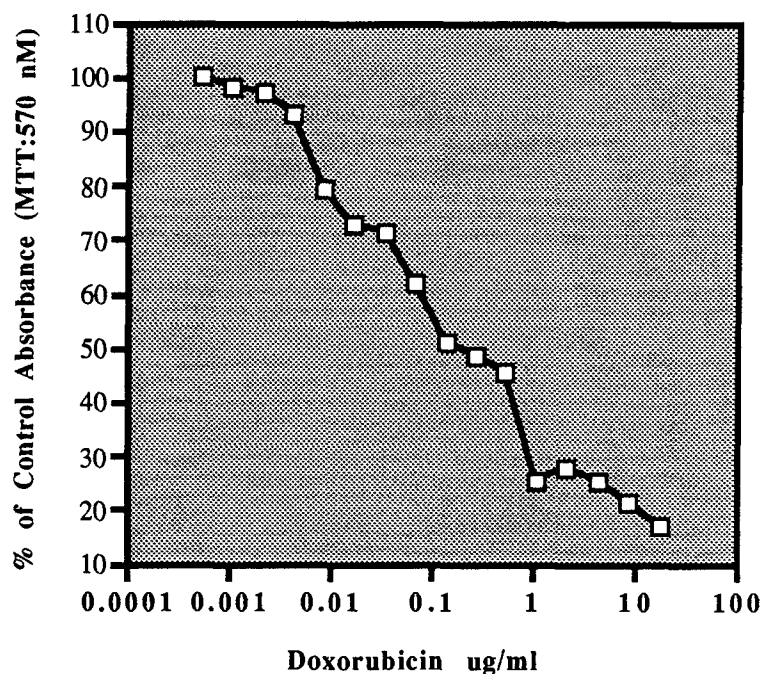
**SK-BR3 Doxorubicin Dose Response
MTT:72 Hour Exposure**



5,000 cells per well were plated in a .1 cc volume in 96 well plates 24 hours prior to the addition of the indicated concentrations of Doxorubicin. Plates were incubated an additional 72 hours prior to the MTT assay. RPMI 1640 was the basic tissue culture media used, with standard supplements.

Figure 4

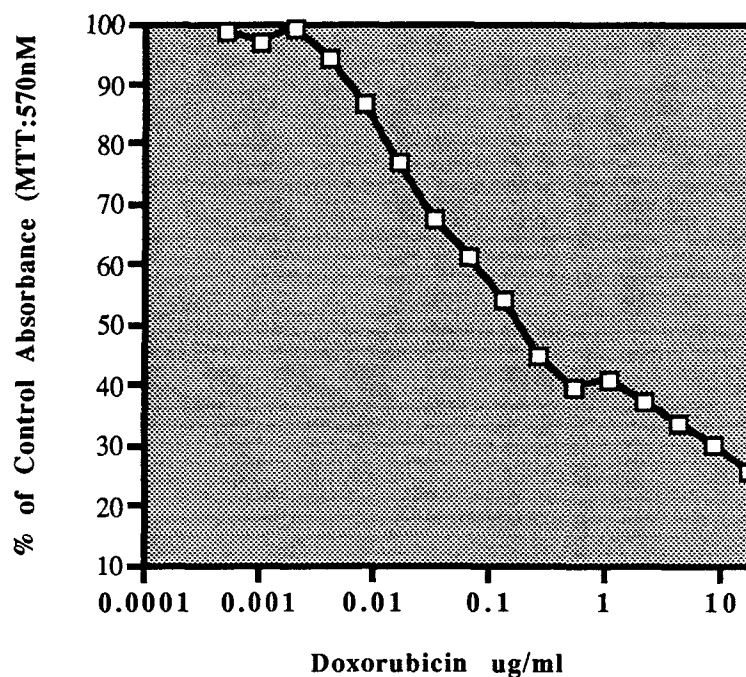
**MDA-MB-231 Doxorubicin Dose Response
MTT:72 Hour Exposure**



5,000 cells per well were plated in a .1 cc volume in 96 well plates 24 hours prior to the addition of the indicated concentrations of Doxorubicin. Plates were incubated an additional 72 hours prior to the MTT assay. RPMI 1640 was the basic tissue culture media used, with standard supplements.

Figure 5

**MCF-7 Doxorubicin Dose Response
MTT:72 Hour Exposure**



5,000 cells per well were plated in a .1 cc volume in 96 well plates 24 hours prior to the addition of the indicated concentrations of Doxorubicin. Plates were incubated an additional 72 hours prior to the MTT assay. RPMI 1640 was the basic tissue culture media used, with standard supplements.

Table 3

(SKBR-3)

Dose of Doxorubicin	Dose of DFO	PIE-A	PIE-M	Observed Inhibition
		%	%	%
.008 µg/ml	5 x10 ⁻⁶ M	28.0	28.0	30.6
.016 µg/ml	5 x10 ⁻⁶ M	36.5	34.1	35.8
.032 µg/ml	5 x10 ⁻⁶ M	50.6	44.3	47.8
.064 µg/ml	5 x10 ⁻⁶ M	64.8	54.5	61.9

Inhibitory effect of combined treatment with a near half-maximal dose of deferoxamine and multiple doses of Doxorubicin (72 hour treatment).

The experiment was conducted in a manner similar to those described above except the target cell line is different and Doxorubicin is the compound employed with Deferoxamine. The inhibitory effect of DFO was 24.1% while the effects of the doses of Doxorubicin were 0%, 8.5%, 22.6%, and 36.8% from smallest to largest dose.

Table 4

(MDA-MB-231)

Dose of Doxorubicin	Dose of DFO	PIE-A	PIE-M	Observed Inhibition
		%	%	%
.008 µg/ml	5 x10 ⁻⁶ M	62	55.9	52.1
.032 µg/ml	5 x10 ⁻⁶ M	76.2	63.2	50.6
.064 µg/ml	5 x10 ⁻⁶ M	89.1	69.6	53.2
.128 µg/ml	5 x10 ⁻⁶ M	100.0	77.1	57.1

Inhibitory effect of combined treatment with a near half-maximal dose of deferoxamine and multiple doses of Doxorubicin (72 hour treatment).

The experiment was conducted in a manner similar to those described above except the target cell line is different and Doxorubicin is the compound employed with Deferoxamine. The inhibitory effect of the DFO was 50.3%. The effects of the doses of Doxorubicin were 11.7%, 25.9%, 38.8%, and 53.9% from the smallest to the largest dose.

Table 5

(MCF-7)

Dose of Doxorubicin	Dose of DFO	PIE-A	PIE-M	Observed Inhibition
		%	%	%
.008 µg/ml	5 x10 ⁻⁶ M	40.1	40.1	43.0
.032 µg/ml	5 x10 ⁻⁶ M	61.4	52.9	44.2
.064 µg/ml	5 x10 ⁻⁶ M	70.0	57.5	45.6
.128 µg/ml	5 x10 ⁻⁶ M	79.2	63.5	50.7

Inhibitory effect of combined treatment with a near half-maximal dose of deferoxamine and multiple doses of Doxorubicin (72 hour treatment).

The experiment was conducted in a manner similar to those described above except the target cell line is different and Doxorubicin is the compound employed with Deferoxamine. The inhibitory effect of the DFO was 40.1%. The effects of the various doses of Doxorubicin were 0%, 21.3%, 29.1%, and 39.1% from the smallest to the largest dose.

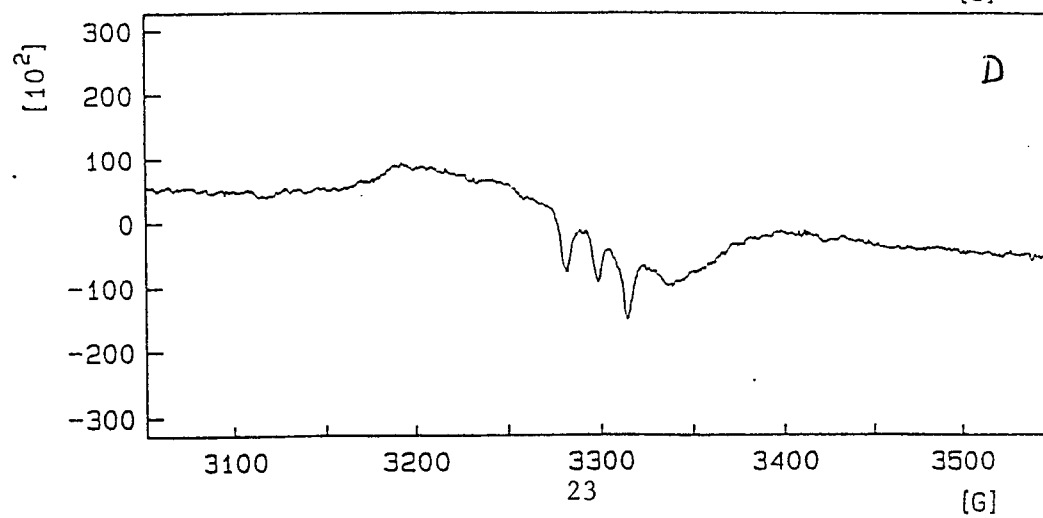
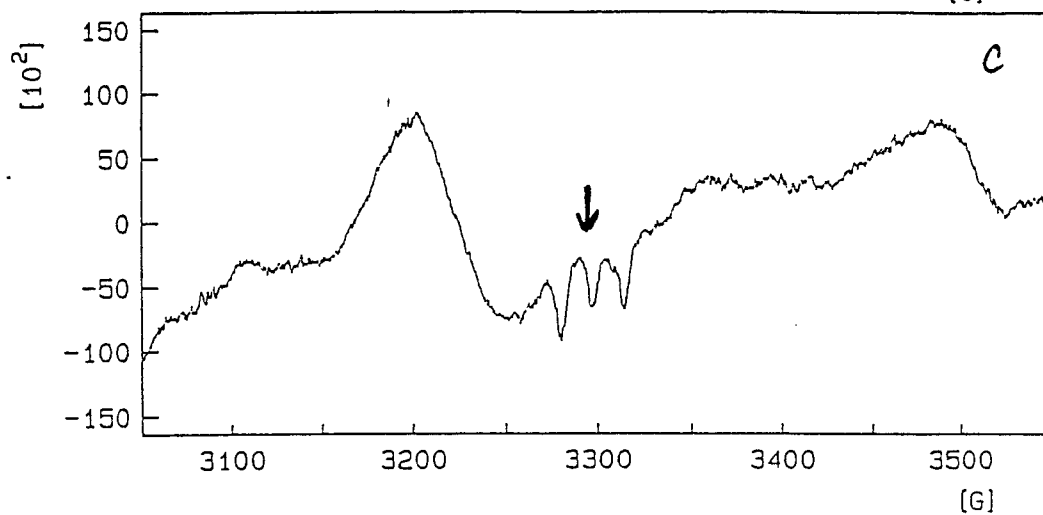
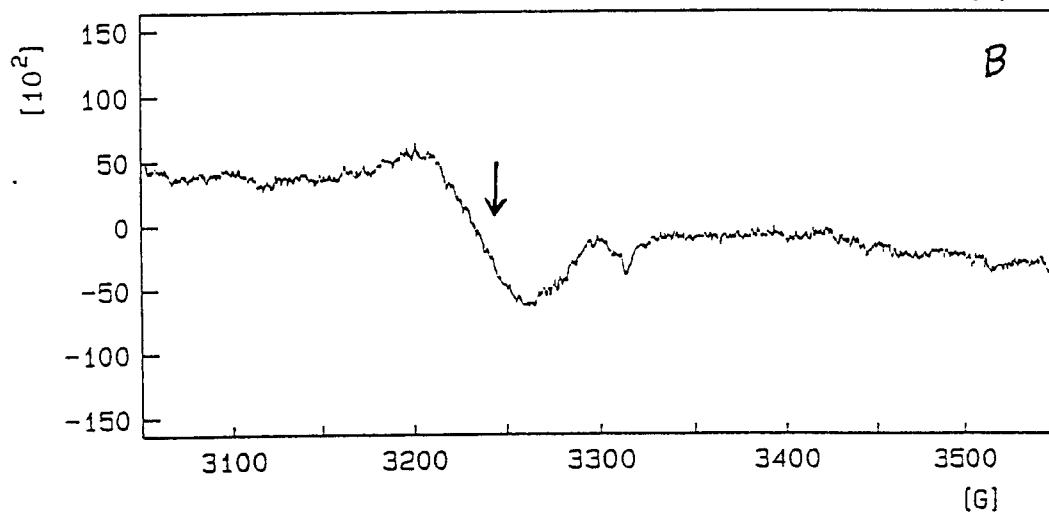
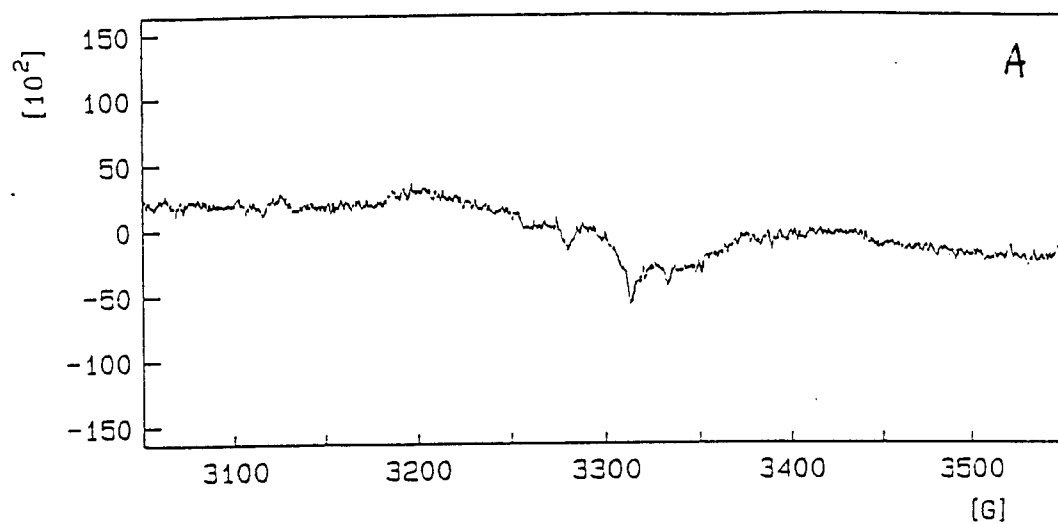
Legend for Figure 6

Whole blood samples were collected from C3H/HEN mice which were either untreated controls, controls exposed to LPS at a dose of 7.5 mg/kg i.p., or animals exposed to combined iron deprivation treatment with HES-DFO (0.5 ml. per day i.p. of a 26 mM solution) and 1.5 mg each of C2 and RL34 i.p. on days 0,3, and 6. Samples were run in the University of Iowa EPR Core Facility on an ESP 300 EPR spectrometer (Bruker Instruments, Karlsruhe, Germany) equipped with an ER035M gaussmeter, ER4111VT variable temperature unit, and EIP-625A microwave frequency counter. Signal averaging from 10 scans was used to improve the signal to noise ratio. All data were collected at 100.0 degrees K. with spectra shown as the normalized average of 10 scans. EPR conditions were receiver gain 5×10^5 , modulation frequency 100 kHz., modulation amplitude 4.027 gauss (G), microwave frequency 9.28 GHz., and microwave power 10 mW.

Panel A is from an untreated control mouse sample. Panel B is representative of the results obtained with 2/3 of the treated mice. Panel C is representative of the results obtained with 1/3 of the treated mice. Panel D is representative of the results obtained with LPS-injected mice.

The arrow in panel B indicates a broad feature that is characteristic of ceruloplasmin. This feature, indicative of significantly elevated plasma ceruloplasmin levels, was found in all samples from mice undergoing iron deprivation treatment but not in either control group. The arrow in panel C indicates a triplet pattern that is characteristic of the nitric oxide-heme radical. This triplet, indicative of significant levels of NO production, was found in the mice given a potentially lethal dose of LPS and in one third of the mice undergoing iron deprivation treatment.

Figure 6



**Direct Evidence That Iron Deprivation Induces
Apoptosis In Murine Lymphoma 38C13**

Jan Kovar^{1,2}, Laura L. Stunz³, Barbara C. Stewart⁴,
Karin Kriegerbeckova², Robert F. Ashman³, John D. Kemp^{1,3,4}

¹Department of Pathology, University of Iowa College of Medicine,
Iowa City, Iowa, U.S.A.

²Institute of Molecular Genetics, Academy of Sciences of the Czech
Republic, Prague, Czech Republic.

³Research Service, Veterans Affairs Medical Center, Iowa City, Iowa.

⁴Pathology Laboratory, Veterans Affairs Medical Center,
Iowa City, Iowa, U.S.A.

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Running Head: Iron deprivation induced apoptosis

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Correspondence and Reprint Requests should be sent to: John Kemp,
Department of Pathology, 5238 Carver Pavilion, University of Iowa Hospitals
and Clinics, Iowa City, IA 52242

Key Words: Iron, apoptosis, transferrin receptor, deferoxamine, lymphoma

Abstract

Prior work suggests that iron deprivation may be capable of inducing apoptosis in tumor cells. We found that the mouse B cell lymphoma 38C13 underwent apoptosis in vitro when deprived of iron by three independent methods: 1) exposure to a synergistic pair of rat IgG monoclonal antibodies against the mouse transferrin receptor, 2) exposure to the iron chelator deferoxamine, and 3) specific deletion of iron from a defined culture medium. When each antibody was present at a concentration of 5 $\mu\text{g/ml}$, the number of living cells declined to approximately 25% after a 24 hour incubation. After 48 hours there were no surviving cells. When deferoxamine was present at a concentration of 10 μM the effects were similar but were delayed by 24 hours. When iron was specifically deleted from the medium, the effects and kinetics were similar to those seen with antibody treatment. For each method of iron deprivation, the reduction in cell viability correlated with the development of apoptosis as assessed by DNA fragmentation analysis and propidium iodide staining. Electron microscopic studies provided additional confirmation of apoptotic cell death. The addition of 500 μM ferric citrate completely prevented apoptosis for each of the three methods of iron deprivation. These studies provide the most direct and compelling evidence yet available to support the view that iron deprivation can specifically induce apoptosis and serve to strengthen the rationale for further study of iron deprivation as a form of cancer treatment.

INTRODUCTION

There is increasing interest in using iron deprivation as a component of cancer therapy and there are three principal means by which iron deprivation can be achieved. One approach employs the iron chelator deferoxamine (DFO). DFO is now under investigation for the treatment of neuroblastoma ^{1,2} and hepatoma ³. A second approach utilizes gallium nitrate. The latter is now under study for the treatment of bladder cancer⁴ and lymphoma ⁵. The third approach is based on the use of monoclonal antibodies (Mabs) against the transferrin receptor. Mabs against the transferrin receptor are active against lymphomas in pre-clinical models ^{6,7}, and are now moving into clinical trials ⁸.

Evidence from experiments with DFO and gallium has been interpreted as supporting the belief that iron deprivation, like many other forms of cancer treatment, can induce apoptosis ⁹⁻¹¹. That evidence, however, is still indirect and incomplete. Thus, even though DFO can induce apoptosis, and even though the effects of DFO are generally reversible by the addition of equimolar quantities of iron salts ¹⁰, it must be kept in mind that DFO will bind other metals when it is present in a concentration which exceeds that of iron ¹². The DFO experiments have therefore not excluded the possibility that chelation of another metal is also a necessary supplemental part of the process that produces apoptosis.

Caution must also be exercised in interpreting the experiments in which gallium treatment is associated with the induction of apoptosis ⁹. Although it is clear that gallium can bind to transferrin instead of iron, and although such binding and the induction of apoptosis can both be reversed by the addition of iron salts ⁹, it must nevertheless be kept in mind that gallium

is thought to interfere with the broader physiological process of endosomal acidification ¹³. That interference not only reduces iron uptake but is also likely to be deleterious to other transport processes. The gallium experiments have therefore not excluded the possibility that reduced uptake of another metal or ligand is a necessary part of the process that produces apoptosis. The data from the DFO and gallium experiments are thus subject to alternative interpretations and are not conclusive with respect to whether iron deprivation alone is sufficient to induce apoptosis.

We have recently shown that iron deprivation inhibits the growth of 38C13 lymphoma in vitro and in vivo ⁷. In the present study, we sought to ascertain whether these treatments inhibited growth of 38C13 cells by virtue of inducing apoptosis. We also sought to ascertain whether specific deletion of iron from a defined culture medium would induce apoptosis.

MATERIALS AND METHODS

Materials.

The cell line producing the rat IgG MAb C2 against the mouse transferrin receptor was described previously ¹⁴. The antibody was purified from rat ascites fluid ⁷. The cell line producing the rat IgG MAb RL34.14.2.5 against the mouse transferrin receptor ¹⁵ was kindly provided by Drs. Jayne Lesley and Ian Trowbridge (Salk Institute, La Jolla, CA). The latter antibody was purified from SCID mouse ascites fluid ⁷. Deferoxamine (Desferal mesylate) was a gift from Ciba-Geigy (Summit, NJ). Human transferrin (substantially iron-free) from Sigma (St. Louis, MO) was rendered iron-saturated as described previously ¹⁶.

Cells.

The mouse B cell lymphoma 38C13 is a well-described, fast-growing cell line ^{17,18}. The cell line was obtained from Dr. George Weiner (University of Iowa City, IA).

Cell culture in defined media.

Cells were cultured in a basic medium supplemented with 5 µg/ml of iron-saturated human transferrin (transferrin medium) or without any iron compound added (iron-free medium). The basic medium was RPMI 1640 containing extra L-glutamine (300 µg/ml), sodium pyruvate (110 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), HEPES (15 mM), ethanolamine (20 µM), ascorbic acid (20 µM), hydrocortisone (5 nM) and 11 trace elements, as described previously ^{16,19}, plus 2-mercaptoethanol (50 µM). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cell survival analysis.

Cells maintained in transferrin medium were harvested by low-speed centrifugation, washed twice with the basic medium, and were then seeded at 20×10^3 cells/100 µl of medium into wells of 96-well plastic plates. Cell survival was evaluated under three iron-depriving conditions: (1) transferrin medium supplemented with the MAbs C2 and RL34.14.2.5 (each at 5 µg/ml), (2) transferrin medium with deferoxamine (10 µM), and (3) iron-free medium. Cell survival was also evaluated when 500 µM ferric citrate was added under each of the iron-depriving conditions. Cell survival was assessed after 24, 48, and 72 hours. The number of living cells was determined by hemacytometer counting after staining with trypan blue.

DNA fragmentation analysis.

Cells grown in transferrin medium were harvested by low-speed centrifugation, washed twice with the basic medium and seeded in plastic culture flasks ($0.5-2 \times 10^6$ cells / 5 ml of medium). Iron-depriving conditions were tested with or without ferric citrate (see "Cell survival analysis"). After 24-72 hours of incubation the cells were harvested by low-speed centrifugation and analyzed as described previously²⁰. Briefly, 10^6 harvested cells per sample were lysed in 400 μ l of lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100). Lysates were centrifuged at 13,000 g for 10 minutes. DNA in the supernatants was precipitated overnight by addition of 400 μ l of isopropanol and 40 μ l of 5 M NaCl. Samples were centrifuged again at 13,000 g for 15 minutes and the pellets dried. Pellets were dissolved in 20 μ l of TE buffer (10 mM Tris, 1 mM EDTA) and 6 μ l of loading dye (40 mM Tris, 20 mM Na acetate, 1 mM EDTA, 50% glycerol, 10% saturated bromophenol blue solution, 1% xylene cyanol). Samples were run on a 1% agarose gel with ethidium bromide (1 μ g/ml) in TAE buffer (40 mM Tris, 20 mM Na acetate, 1 mM EDTA) at 5-10 V/cm. DNA was visualized under UV light and photographed.

PI staining analysis.

Cells previously grown in transferrin medium were harvested by low-speed centrifugation, washed twice with the basic medium and seeded in plastic culture flasks ($1-2 \times 10^6$ cells/5 ml of medium). Iron-depriving conditions were tested with or without ferric citrate (see "Cell survival analysis"). After a 24-hour incubation the cells were harvested by low-speed centrifugation and stained. Briefly, approximately 4×10^6 cells per sample were washed twice with 2 ml of PBS and then fixed in 2 ml of 70% ethanol at

4°C overnight. The cells were centrifuged and washed with 2 ml of PBS. Cell pellets were treated with 1 ml of 0.1% Triton X-100 at 4°C for 3 minutes and centrifuged. Supernatants were removed and 1 ml of RNase (100 units/ml) was added. After a 10-minute incubation at room temperature the samples were spun and supernatants aspirated. Propidium iodide (1 ml, 50 µg/ml) was added and the samples were incubated in the dark at 4°C overnight. Stained cells were analyzed on a Becton Dickinson FACSCAN (Mountain View, CA).

Electron microscopic analysis.

Cells grown in transferrin medium were harvested and washed as described above (see "Cell survival analysis"). Washed cells were seeded at 4×10^6 cells/20 ml of medium in plastic culture flasks. Cells were cultured in either iron-free medium or transferrin medium. After a 24-hour incubation the cells were harvested by low-speed centrifugation and prepared for electron microscopy. The cell pellets were fixed overnight with 2.5% glutaraldehyde in a 0.1 M cacodylate buffer and then rinsed in the buffer for 30 minutes. The pellets were then post-fixed with 1% osmium tetroxide in cacodylate buffer for 90 minutes, rinsed in the buffer for 30 minutes, and were then dehydrated through graduated alcohols and propylene oxide. The pellets were then embedded in epoxy resin and sections were cut with a Reichert-Jung Ultracut E ultra-microtome. The sections were stained with uranyl acetate and lead citrate and were visualized with a Philips CM10 transmission electron microscope.

RESULTS

Kinetics of cell death induced by iron deprivation.

In order to ascertain whether the induction of apoptosis was involved in the anti-tumor effects we had previously observed with iron deprivation treatments *in vitro* and *in vivo* ^{7,21}, we began our studies with a kinetic analysis of the effects of various iron deprivation treatments on the 38C13 lymphoma *in vitro*.

We therefore measured the effect on cell survival after cells were transferred into: (1) transferrin medium with the MAbs C2 and RL34.2.5 (5+5 $\mu\text{g/ml}$), (2) transferrin medium with DFO (10 μM), and (3) iron-free medium (Figure 1). The cells incubated with the MAbs and in iron-free medium behaved in a similar way. The number of surviving cells declined from the original $20 \times 10^3/\text{well}$ to approximately $5 \times 10^3/\text{well}$ and $2 \times 10^3/\text{well}$, respectively, within the first 24 hours. After 48 hours of incubation the number of surviving cells was virtually zero in both cases. In the case of cells incubated with DFO, however, a different kinetic pattern was observed. For the first 24 hours growth was similar to that of control cells incubated in transferrin medium alone. The number of living cells increased to approximately $35 \times 10^3/\text{well}$. Then, after 48 hours, the number of surviving cells declined to approximately $8 \times 10^3/\text{well}$. Meanwhile, the control cells grew to about 200×10^3 cells/well. After 72 hours the number of surviving cells in the DFO treated group was also nearly zero.

We also asked whether ferric citrate could overcome the effects of the iron-depriving conditions. For all the iron-depriving conditions employed, the

addition of 500 μ M ferric citrate completely blocked cell death and the cells grew at a rate similar to that of the control cells.

Induced cell death is associated with DNA fragmentation.

To evaluate the development of apoptosis we analyzed 38C13 cells for DNA fragmentation by agarose gel electrophoresis. As before, we tested the effect of: (1) transferrin medium with the MAbs C2 and RL34.2.5 (5+5 μ g/ml), (2) transferrin medium with DFO (10 μ M), and (3) iron-free medium. We also asked whether 500 μ M ferric citrate was capable of reversing the effect of the iron-depriving conditions.

After a 24-hour incubation either with the MAbs or with iron-free medium we detected cleavage of 38C13 DNA into oligonucleosomal fragments (Figure 2A). The addition of ferric citrate completely blocked the DNA fragmentation. DNA fragmentation was not detected in the cells incubated with DFO for 24 hours. After 72 hours, however, the cells incubated with DFO did produce a characteristic ladder pattern and the fragmentation was again blocked by the addition of ferric citrate (Figure 2B).

Induced cell death is associated with the accumulation of cells with hypodiploid DNA content.

As an additional method to evaluate the development of apoptosis we asked whether 38C13 cells would display a hypodiploid peak by flow cytometric analysis under conditions of iron deprivation. The iron-depriving conditions tested were the same as those employed in DNA fragmentation analysis; i.e., (1) transferrin medium with the MAbs C2 and RL34.2.5 (5+5

μM), (2) transferrin medium with DFO ($10\ \mu\text{M}$), and (3) iron-free medium. As before, we also asked whether ferric citrate ($500\ \mu\text{M}$) would abrogate the effect of the iron-depriving conditions.

As was the case with DNA fragmentation, after a 24-hour incubation either with the MAbs or with iron-free medium we detected the accumulation of cells with hypodiploid DNA content characteristic of apoptosis in conjunction with a reduction in the proportion of cells traversing the cell cycle (Figure 3). The addition of ferric citrate once again completely abrogated the effect of the MAbs and of iron-free medium. The accumulation of hypodiploid cells became detectable in the DFO treated group after 48 hours (data not shown).

Induced cell death is associated with ultrastructural changes characteristic for apoptosis.

As a third method to evaluate the development of apoptosis, we examined changes in ultrastructural features of 38C13 cells induced by iron deprivation. Cells were incubated in iron-free medium or in transferrin medium with the MAbs C2 and RL34.2.5 ($5+5\ \mu\text{g/ml}$).

After a 24-hour incubation in iron-free medium, transmission electron microscopy revealed typical apoptosis-associated ultrastructural changes. These changes included vacuolization, disappearance of microvilli, chromatin condensation, and nuclear fragmentation (Figure 4). Similar ultrastructural changes were found for cells that were incubated for 24 hours with the MAbs (data not shown).

DISCUSSION

Prior work has been interpreted to indicate that iron deprivation can induce apoptosis in tumors ⁹⁻¹¹. However, even though the evidence provided has been strongly suggestive, it has nevertheless been indirect and inconclusive. Thus, although Fukuchi et al. showed that DFO treatment generated apoptosis in HL60 cells ¹¹, they did not describe reversal of the effect with iron salts and also did not describe an equivalent effect with iron deprivation per se. In addition, while Hileti et al. showed that presaturation of iron chelators with ferric chloride could prevent the induction of apoptosis in HL60 cells and activated T cells ¹⁰, and Riaz-Ul-Haq et al. showed that ferric ammonium citrate would block the induction of apoptosis in CCRF-CEM cells by gallium ⁹, both groups again did not describe an equivalent effect with iron deprivation per se.

The studies presented here provide direct evidence that iron deprivation is in itself sufficient to induce apoptosis in neoplastic cells. A critical feature of the work was that the cell line used was capable of growth in a completely defined culture medium ^{16,19}. This allowed us to perform an experiment in which iron was specifically deleted, and it was then observed that the 38C13 lymphoma cells underwent rapid, essentially complete, apoptotic cell death. Although this finding directly demonstrates that iron deprivation per se can induce apoptosis, it does not prove that iron deprivation is the only means by which iron chelators, gallium, and Mabs against the transferrin receptor produce apoptosis.

The finding that a pair of IgG Mabs against the transferrin receptor was capable of inducing apoptosis in 38C13 cells in the presence of transferrin-bound iron is both novel and consistent with our prior finding that

the same pair of antibodies produced synergistic growth inhibition of 38C13 in vitro and frequent regression of established 38C13 tumors in vivo ⁷.

Although we do not yet fully understand why the antibodies are synergistic, we are currently investigating the hypothesis that they produce a greater degree of receptor down-modulation than the single Mab ²¹ and that the resulting additional decrement in iron uptake is sufficient to rapidly cross a critical threshold of iron deprivation ²².

The finding that the induction of apoptosis with DFO treatment was delayed by 24 hours when compared either to the Mab treatment or to specific deletion of iron is clearly consistent with the prior studies of Fukuchi et al. ¹¹, Hileti et al. ¹⁰, and Riaz-Ul-Haq et al. ⁹. The reproducibility of this observation continues to support the views that DFO enters cells slowly, most likely by pinocytosis ²³, and that DFO does not remove iron from transferrin to any significant degree at physiologic pH but rather binds iron only after it is released at low pH in the endosome ²⁴. Thus, DFO appears to have only limited access to cellular iron storage pools and drains iron from cells in a relatively slow manner.

The present studies are interesting to consider in light of the uncertainty surrounding the physiological significance and in vivo relevance of transferrin independent iron uptake ²⁵⁻³². On the one hand, 38C13 provides an additional, unequivocal example of a cell line that can grow in the absence of transferrin (but not iron) in vitro. Thus it is iron, and not necessarily transferrin dependent delivery of iron, that is critical for growth in vitro. This fact was further emphasized by the observation that when iron was present in the form of ferric-transferrin and when transferrin receptor function was blocked by the Mab pair (and the cells were thus destined to undergo apoptosis), they were completely rescued by the addition of ferric citrate.

On the other hand, we observed in recent studies that the same Mab pair was capable of causing a significant fraction of established tumors to undergo regression in vivo ⁷. Such a result clearly would not have been expected if the tumor cells had had access to significant quantities of non-transferrin bound iron. The observation of regression is thus consistent with the fact that nearly all iron is normally bound by transferrin in vivo and, by extension, that tumors are obligated to make use of their transferrin receptors in order to obtain the iron necessary for growth. The fact that not all of the tumors regressed, however, might mean that there was enough non-transferrin bound iron available in the tumor microenvironment to permit some tumor escape. Alternatively, and perhaps more likely, it may have been that the level of penetration of the antibody in the tumor bed in vivo was inadequate to produce a degree of receptor saturation equivalent to that produced in vitro. Clearly, more work will be required in order to definitively ascertain whether tumors are somehow exposed to, and take up, physiologically significant quantities of non-transferrin bound iron in vivo.

A variety of cellular insults or stresses are now believed to be capable of activating pathways that lead to apoptosis. Two may be of particular interest for future research into the mechanisms underlying iron deprivation induced apoptosis. One pathway of potential interest involves the induction of p53 expression as a result of DNA damage ³³. The p53 pathway may be relevant because iron deprivation is known to be associated with accumulation of single strand breaks in DNA ³⁴. Such breaks are thought to occur because iron deprivation inhibits the activity of ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis ^{35,36}. A second apoptotic pathway of potential interest is that which occurs as a result of hypoxic stress. This pathway may be relevant because iron deprivation, like

hypoxia, can inhibit electron transport chain function and thus decrease oxidative phosphorylation ³⁷. In addition, iron deprivation also appears to activate genes, such as the erythropoietin gene, that are known to be activated by hypoxia ^{38,39}. Although little is known about how hypoxia induces apoptosis, it is believed that Bcl-2 and Bcl-xL oppose the process ^{40,41}.

Since there is rising interest in the use of iron deprivation as a form of cancer treatment ¹⁻⁸, and since it now appears to be very clear that iron deprivation (like several other cancer treatment modalities) exhibits the capacity to induce apoptosis in tumors, then the justification for further studies of iron deprivation and its mechanism(s) of action has been strengthened.

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Figure Legends

Fig. 1. Effect of iron-depriving conditions on survival of 38C13 cells. Three iron-depriving conditions were tested: (1) transferrin medium supplemented with MAbs C2 and RL34.14.2.5 against the transferrin receptor, each at 5 $\mu\text{g/ml}$ (Tf & C2+RL), (2) transferrin medium with 10 μM deferoxamine (Tf & DFO), and (3) iron-free medium (-Fe). Control cells were incubated in transferrin medium (Tf) alone. A matching set of four groups was also tested in which 500 μM ferric citrate was added to each of the four groups just described. Each column represents the mean of three separate cultures $\pm\text{SEM}$. Cells were seeded at 20×10^3 cells/100 ml of medium in the well. The number of living cells was determined after 24 hours and after 48 hours of incubation.

Fig. 2. Effect of iron-depriving conditions on the induction of DNA fragmentation in 38C13 cells. The same three iron-depriving conditions were tested: (1) transferrin medium with MAbs C2 and RL34.14.2.5 against the transferrin receptor, each at 5 $\mu\text{g/ml}$ (Tf & C2+RL), (2) transferrin medium with 10 μM deferoxamine (Tf & DFO), and (3) iron-free medium (-Fe). Control cells were incubated in transferrin medium (Tf) alone. A matching set of four groups was also tested in which 500 μM ferric citrate was added to each of the four groups just described. The control DNA ladder was a 1 kb DNA ladder from Gibco. Cells were seeded either at 2×10^6 cells/5 ml of medium (A), or at 0.5×10^6 cells/5 ml of medium (B). DNA fragmentation was determined after 24 hours (A) or 72 hours (B) of incubation.

Fig.3. Effect of iron-depriving conditions on DNA histograms of 38C13 cells stained with propidium iodide. The same three iron-depriving conditions were tested: (1) transferrin medium with Mabs C2 and RL34.14.2.5 against the transferrin receptor, each at 5 $\mu\text{g/ml}$ (Tf & C2+RL), (2) transferrin medium with 10 μM deferoxamine (Tf & DFO), and (3) iron-free medium (-Fe). Control cells were incubated in transferrin medium (Tf) alone. A matching set of four groups was also tested in which 500 μM ferric citrate was added to each of the four groups just described. Cells were seeded at 2×10^6 cells/5 ml of medium in culture flask. After a 24 hour incubation the cells were stained with propidium iodide and analyzed by flow cytometry.

Fig. 4. Effect of iron-free medium (B) on ultrastructural features of 38C13 cells. Control cells were incubated in transferrin medium (A). Cells were seeded at 4×10^6 cells/20 ml of medium and, after a 24 hour incubation, the cells were harvested and prepared for electron microscopy. Magnification is 5900x.

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Figure 1

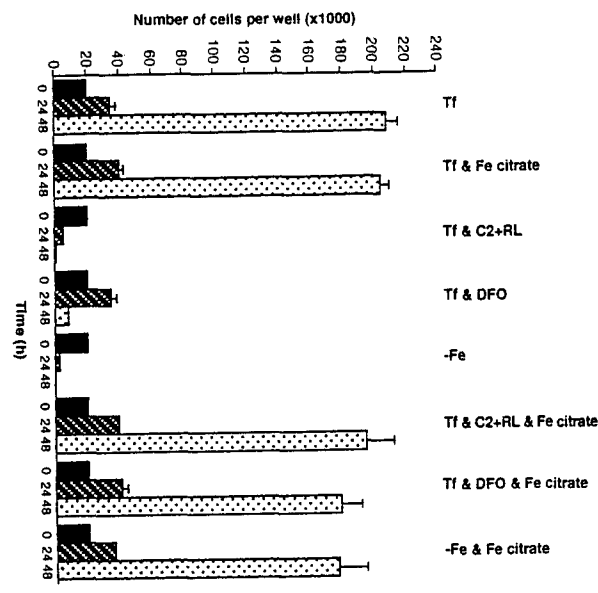


Figure 2

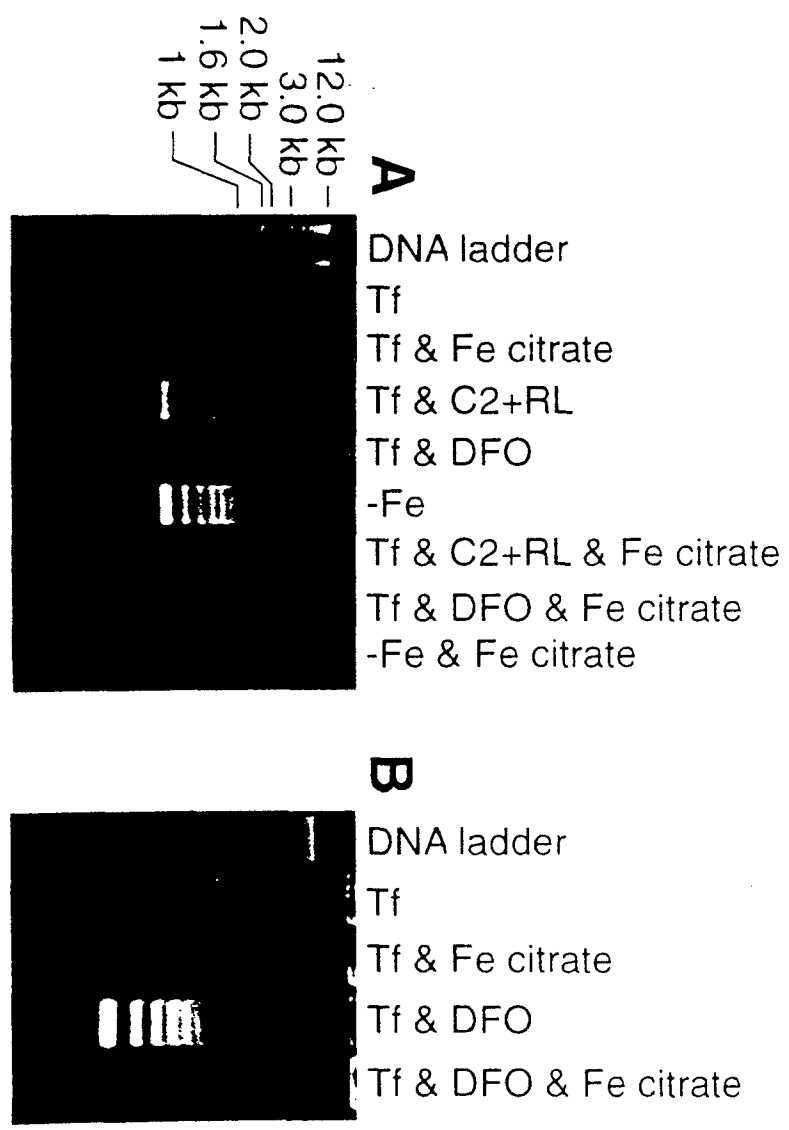


Figure 3

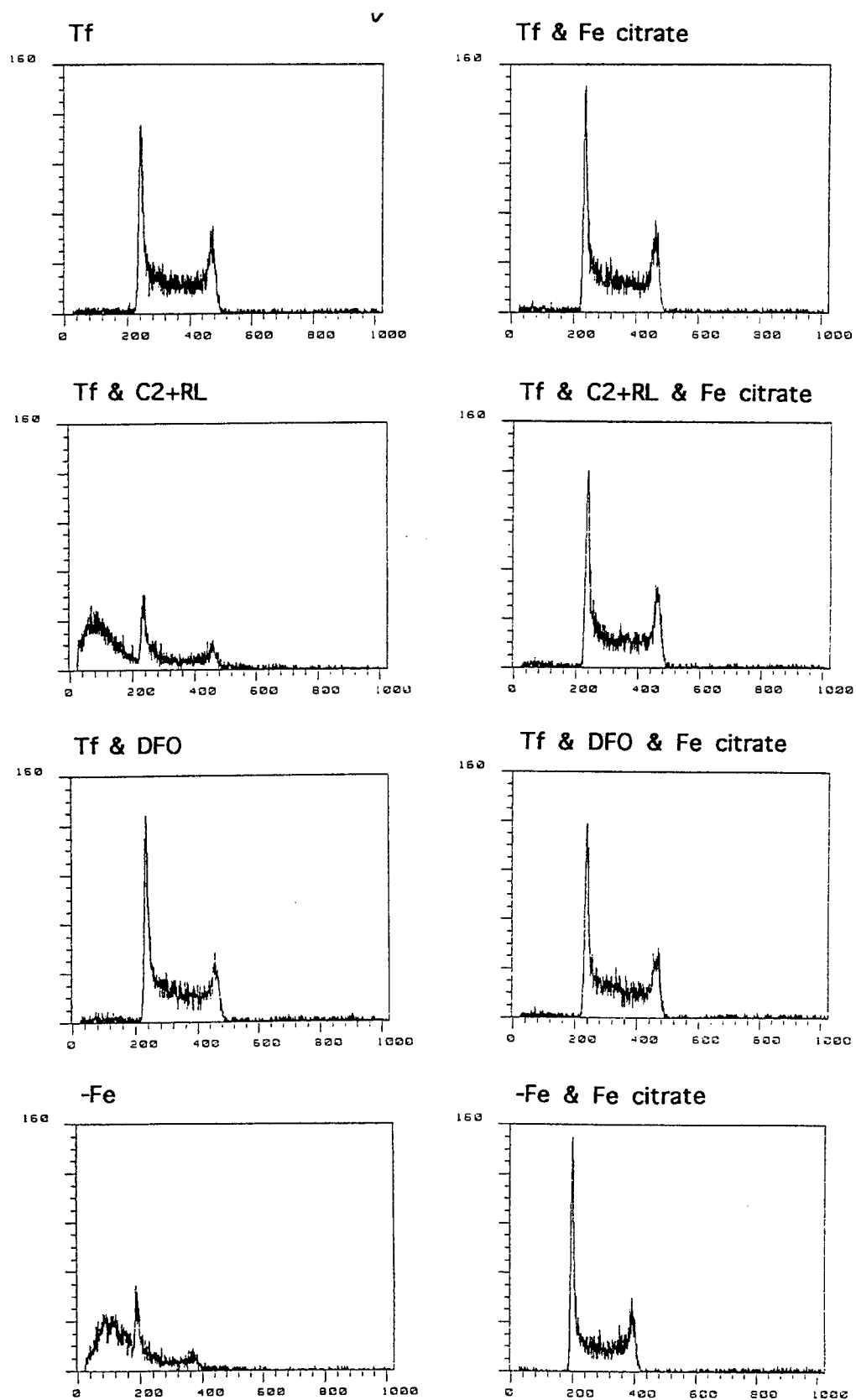
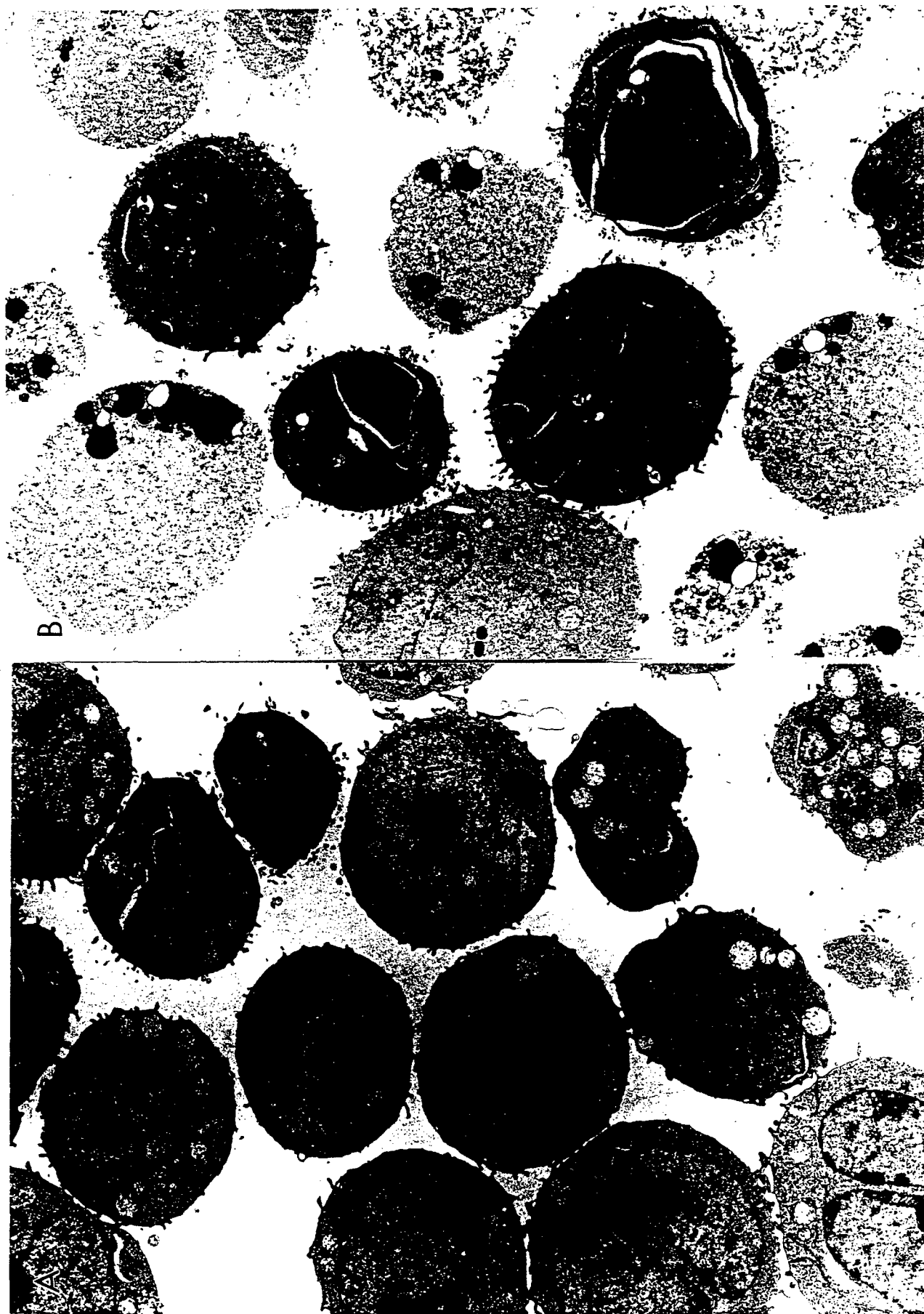


Figure 4





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Phyllis Rinehart
PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management