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Differential Regulation of Iron Regulatory Element-binding Protein(s) in Cell Extracts of Activated Lymphocytes *Versus* Monocytes-Macrophages*

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The intracellular iron level exerts a negative feedback on transferrin receptor (TfR) expression in cells requiring iron for their proliferation, in contrast to the positive feedback observed in monocytes-macrophages. It has been suggested recently that modulation of TfR and ferritin synthesis by iron is mediated through a cytoplasmic protein(s) (iron regulatory element-binding protein(s) (IRE-BP)), which interacts with ferritin and TfR mRNA at the level of hairpin structures (IRE), thus leading to inhibition of transferrin mRNA degradation and repression of ferritin mRNA translation.

In the present study we have evaluated in parallel the level of TfR expression, ferritin, and IRE-BP in cultures of: (i) circulating human lymphocytes stimulated to proliferate by phytohemagglutinin (PHA) and (ii) circulating human monocytes maturing *in vitro* to macrophages. The cells were grown in either standard or iron-supplemented culture. TfR and ferritin expression was evaluated at both the protein and mRNA level. IRE-BP activity was measured by gel retardation assay in the absence or presence of β-mercaptoethanol (spontaneous or total IRE-BP activity, respectively).

Spontaneous IRE-BP activity, already present at low level in quiescent T lymphocytes, shows a gradual and marked increase in PHA-stimulated T cells from day 1 of culture onward. This increase is directly and strictly correlated with the initiation and gradual rise of TfR expression, which is in turn associated with a decrease of ferritin content. Both the rise of TfR and spontaneous IRE-BP activity are completely inhibited in iron-supplemented T cell cultures. In contrast, the total IRE-BP level is similar in both quiescent and PHA-stimulated lymphocytes, grown in cultures supplemented or not with iron salts.

Monocytes maturing *in vitro* to macrophages show a sharp increase of spontaneous and, to a lesser extent, total IRE-BP; the addition of iron moderately stimulates the spontaneous IRE-BP activity but not the total one. Here again, the rise of spontaneous IRE-BP from very low to high activity is strictly related to the parallel increase of TfR expression and, suprisingly, also with a very pronounced rise of ferritin expression observed at both the mRNA and protein level.

It is noteworthy the effect of β-mercaptoethanol is cell specific, *i.e.* the ratio of total *versus* spontaneous IRE-BP activity is different in activated lymphocytes and maturing monocytes.

In conclusion, our studies provide evidence that the levels of spontaneous IRE-BP activity and TfR expression are directly correlated in both a cell proliferation model (PHA-stimulated T lymphocytes) and a cell differentiation model (monocytes maturing to macrophages). Furthermore, both parameters are either down- or up-modulated by iron in lymphocytes or monocytes-macrophages, respectively. The linkage between TfR expression and spontaneous IRE-BP activity strongly suggests that this binding protein plays a crucial role in the modulation of TfR expression.

In higher eukaryotes, the transferrin receptor (TfR)[1] and ferritin mediate the uptake and detoxification of iron, respectively. Iron is delivered to most cells via endocytosis of diferric-transferrin bound to the TfR (1). In the endosome, iron is released from transferrin and transferred to the cytosol. Once in the cytoplasm, iron is used either for metabolic processes or sequestered in ferritin, a hollow spherical molecule composed of 24 subunits encoded by two highly homologous genes (H and L) (2).

The expression of TfR is regulated by complex mechanisms. A variety of factors including hemin, protoporphyrin IX, and growth factors modulate its rate of synthesis (1). It is generally conceded that the expression of TfR is directly correlated with the rate of cell growth (3, 4) and inversely related to the amount of iron accumulated in the cells (5–8). The relationship with cell growth is at least in part explained by the absolute requirement for iron of the enzyme ribonucleotide reductase, which converts ribonucleotides to their deoxy derivates (9, 10). The iron modulation has been clearly demonstrated by experiments of iron load or chelation in fibroblasts (5), leukemic cell lines (6, 8), and mitogen-activated T lymphocytes (7). In cultured human monocytes-macrophages, however, iron up-regulates TfR expression, thus is in sharp contrast to the negative feedback reported in the other cell types (11). Apparently, the iron load down-modulates TfR expression in cells utilizing iron to sustain cellular proliferation (*i.e.* activated T lymphocytes) whereas it up-regulates TfR in cells physiologically involved in the storage of this element (*i.e.* monocytes-macrophages). Interestingly, in both

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[1] The abbreviations used are: TfR, transferrin receptor; IRE, iron regulatory element; IRE-BP, IRE-binding protein; BME, β-mercaptoethanol; PHA, phytohemagglutinin; kb, kilobase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H, heavy; L, light.

cell types iron load leads to a marked stimulation of ferritin synthesis (2).

Recent studies have shed light on the molecular basis for the coordinate regulation of TfR and ferritin expression. The 5′-untranslated region of ferritin mRNA and the 3′-untranslated region of TfR mRNA contain a similar *cis*-acting RNA element, termed the *iron regulatory element* (IRE) (12–15), which interacts with a common IRE-binding protein(s) (IRE-BP) (16–22). On a theoretical basis, IRE and its BP can account for the coordinate iron-dependent regulation of both TfR and ferritin expression (23–25); thus, as cellular iron becomes limiting, a greater fraction of IRE-BP is recruited into a high affinity binding state (26). The high affinity interaction between the IRE-BP and the IRE in the ferritin and TfR mRNA leads to repression of translation of ferritin mRNA (14, 21, 27) and correlates with the inhibition of TfR mRNA degradation (28, 29). Thus, the binding of the IRE-BP to IRE may coordinately regulate a decrease in ferritin biosynthesis and an increase in that of TfR. Interestingly, recent studies have shown that the affinity status of the IRE-BP is regulated through oxidation-reduction mechanisms (30); thus, the IRE-BP present in cell extracts derived from different cell types is usually activated *in vitro*, although to a different extent, by the addition of β-mercaptoethanol (BME).

In an attempt to verify this hypothetical model, we have comparatively evaluated the level of TfR expression and IRE-BP activity in cultures of PHA-stimulated T lymphocytes and monocytes-macrophages, supplemented or not with iron.

EXPERIMENTAL PROCEDURES

Materials

Human transferrin (Sigma) was purified by gel filtration chromatography to an electrophoretically homogeneous preparation, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ferric ammonium citrate and bovine serum albumin (fraction V) were purchased from Sigma. ^{125}I-Transferrin was obtained from Du Pont-New England Nuclear.

Isolation and Culture of Monocytes and Lymphocytes

Peripheral blood mononuclear cells were obtained from 18–40-year-old healthy male and female donors. Venous blood mixed with 12 IU/ml sodium heparinate was separated on Ficoll-Hypaque solution (Pharmacia LKB Biotechnology Inc.) at 450 × *g* for 20 min. Cells were washed three times with RPMI 1640 medium (Flow Laboratories, Inc., Arvine, Scotland) and then suspended (4×10^6 cells/ml) in the same medium containing 20% fetal calf serum (Flow Laboratories). Monocytes were separated from lymphocytes by adherence (1 h at 37 °C) in plastic dishes. After removal of the non-adherent population cells were washed three times with RPMI 1640 medium to remove residual nonadherent cells. Cytochemical and surface marker analyses revealed that the adherent population consisted of > 95% monocytes. Thereafter, cells were cultured in RPMI 1640 medium containing 15% fetal calf serum in 5% CO_2 in air at 37 °C. At different culture days monocytes-macrophages were harvested by vigorous pipetting after a 20–30-min incubation at 4 °C. Cell viability ranged from 85 to 100%, as determined by the trypan blue exclusion test. Nonadherent cells, largely composed of T lymphocytes, were grown in RPMI 1640 medium containing 10% fetal calf serum and 5 μg/ml PHA (Sigma) in 5% CO_2 in air at 37 °C. The T lymphocyte proliferation induced by PHA was evaluated by [^{3}H] thymidine incorporation assay as described previously (31).

TfR Assay

Purified human transferrin was conjugated by the solid phase lactoperoxidase method (Du Pont-New England Nuclear radioiodination system) as described (16). The binding reaction was performed in polypropylene tubes (12 × 75 mm) in Hanks' saline medium containing 0.1% bovine serum albumin (5×10^6 cells/ml, labeled and unlabeled transferrin, 500 ng and 1 mg/ml, respectively). Unbound ligand was removed by passing the cells through a density cushion (7, 31).

Two hundred microliters of the cell suspension was incubated and layered on 150 μl of a dibutyl and dinoyl phtalate mixture (Merck) up to a final density of 1.205 in 400-μl plastic tubes and then centrifuged in an Eppendorf microcentrifuge (13,000 × *g*/2 min). The resulting supernatant and a major part of the phtalate cushion were then aspirated. The tips of the vials, containing cell pellets, were severed with a scalpel, transferred to plastic vials, and the radioactivity in the cell pellet was counted. "Total" binding corresponds to the radioactivity in the cell pellet, and "nonspecific" binding represents radioactivity detected in the presence of a large excess of unlabeled Trf. "Specific" binding data are the differences between total and nonspecific values.

Before binding, cells were washed three times in 40 ml of Hanks' saline solution. The number of washes did not modify the Trf binding capacity because cells were grown in fetal calf serum and bovine Trf, the latter having a low affinity for human TrfR (32).

Ferritin Content

The intracellular concentration of ferritin was evaluated by a radioimmunoassay, using a kit from Hoechst (Behring Institut, Frankfurt am Main, Federal Republic of Germany). Samples for ferritin determination were lysed in distilled H_2O, freeze-thawed three times, and centrifuged at 13,000 × *g*, 15 min at 4 °C; the supernatant was used for radioimmunodetermination of ferritin.

Protein concentration was determined by the dye binding method (Bio-Rad).

RNA Analysis

Total RNA was extracted from $2–3 \times 10^7$ macrophages or 1×10^8 lymphocytes by the guanidine isothiocyanate phenol-chloroform technique (33). Integrity of RNA was assessed by evaluation of the 28 S/18 S RNA ratio on 1% agarose minigels stained with ethidium bromide. The 9.5–0.3-kb RNA ladder was used as molecular size marker. The levels of TfR and ferritin RNA were evaluated by dot and Northern blot analyses. For dot blot analysis, decreasing amounts of total RNA (4–0.5 μg) were dotted onto nitrocellulose paper (BA-85, Schleicher & Scheull) through a 96-well minifold apparatus, fixed by baking for 2 h at 80 °C in a vacuum, and hybridized to 10^7 cpm of nick-translated probe (specific activity, $3–6 \times 10^8$ dpm/μg) as described (34). Filters were washed under stringent conditions (15 mM NaCl, 1.5 mM sodium citrate, 0.1% sodium dodecyl sulfate, pH 7.0, at 65 °C) and exposed at −70 °C to Kodak SO-282 x-ray film using X-Omatic intensifying screens. For Northern analysis, 5 μg of total RNA was run on 1% formaldehyde gels, transferred onto nitrocellulose filter by capillary blot (34), hybridized, washed, and exposed as for dot blots. Rehybridization was carried out after removal of probe by washing with 5 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.002% Ficoll (Pharmacia), 0.002% polyvinylpyrrolidone (Sigma), and 0.002% bovine serum albumin (Sigma) for 1 h at 65 °C. Appropriately exposed autoradiograms were analyzed by densitometric scanning. Expression of H- and L-ferritin genes was evaluated by densitometric comparison with standard dots of 4 μg of human rRNA containing 1–250 pg of the human H- and L-ferritin cDNA probes. Appropriate conversion factors were formulated for each probe, thus accounting for their size as compared with full-length mRNA. The final data are expressed as pg of mRNA/μg of total RNA dotted onto the filters.

The probes used were the 4.9-kb full-length cDNA of human TrfR (pcD-TR1) (35, 36), the 0.86- and 0.70-kb cDNA of human H- and L-ferritin chains (FR 33 and FR 36) (37), and the 11-kb genomic probe of human 28 S ribosomal RNA (pHR 28A).

Evaluation of IRE-BP

Preparation of Cytoplasmic Extracts—$5–10 \times 10^6$ lymphocytes or monocytes were lysed at 4 °C in 0.2 ml of buffer solution (10 mM HEPES, pH 7.50, 3 mM $MgCl_2$, 40 mM KCl, 5% glycerol, and 1 mM dithiothreitol supplemented with 0.2% Nondiet P-40). After lysis, samples were diluted with extraction buffer, thus providing a final protein concentration of approximately 50 μg/ml. Nuclei were removed by centrifugation at 10,000 × *g* for 1 min and the supernatant stored at −80 °C.

The protein concentration was determined on aliquot samples from the soluble supernatants using the Bio-Rad protein assay.

For the IRE-BP assay, 20 μl of cellular extracts (corresponding to 1 μg of total proteins) was used. For full activation of IRE-BP, 2 μl of 20% BME was added for 10 min just prior to the assay.

Generation of in Vitro Transcripts—An oligonucleotide sequence corresponding to the 5′ IRE of human ferritin heavy chain mRNA, bases 31–58 (38), has been subcloned between the *Eco*RI and *Sma*I site of the pSPT18 vector (Boehringer Mannheim) (16). This plasmid, pSPT-fer, was linearized at the *Bam*HI site and transcribed *in vitro* with T7 RNA polymerase to generate a radiolabeled RNA of 46 bases.

The transcription reaction was performed at 40 °C for 1 h with 1 μg of plasmid DNA in the presence of 100 mCi of [α-^{32}P]CTP (800 Ci/mmol) (Amersham Corp.) and 0.5 mM ATP, GTP, and UTP (Boehringer Mannheim) in a 20-μl reaction volume. Unincorporated nucleotides were removed on a Sephadex G-50 column. The specific activity of transcripts under these conditions is 1.3×10^9 dpm/μg RNA.

Gel Retardation Assay—Analyses of RNA-protein interactions were performed as described by Leibold and Munro (16) by incubating 0.2 ng of ^{32}P-labeled RNA transcript with 1-μg aliquots from cytoplasmic protein fractions in a 20-μl reaction volume. Under these conditions the RNA was found to be in molar excess over IRE-BP.

Binding was carried out for 10 min at room temperature, and heparin was added to a final concentration of 5 mg/ml for another 10 min. RNA-protein complexes were resolved in 4% nondenaturing gels as described (39).

To determine the total amount of IRE-BP, samples were reduced with 2% BME prior to the assay.

RESULTS

TfR Expression and IRE-BP Activity in Mitogen-activated T Lymphocytes—Peripheral blood lymphocytes, grown in the presence of PHA and the absence or presence of iron salts, were harvested at 24-h intervals to evaluate the expression of TfR at both protein (^{125}I-transferrin binding assay) and RNA levels (Northern blot analysis). Control experiments showed that the addition of iron salts induced a slight increase in the level of [^{3}H]thymidine incorporation by PHA-stimulated lymphocytes (Fig. 1). Furthermore, the addition of ferric ammonium citrate elicited a 3–4-fold decrease in the transferrin binding capacity of PHA-stimulated T lymphocytes (Fig. 1). Finally, the addition of iron salts induced a pronounced inhibition of TfR RNA expression. Thus, no TfR RNA was detected in control fresh lymphocytes; however, starting from day 1 of culture, the lymphocytes showed a gradual rise of the level of the 5.1-kb TfR RNA transcript up to peak expression on day 3 (Fig. 1). At all culture times, cells treated with ferric ammonium citrate exhibited markedly lower levels of TfR RNA as compared with controls (Fig. 1). We also analyzed the expression of IRE-BP in these cells at different days of culture by gel retardation assays before and after treatment of the cytoplasmic extracts with the reducing agent BME; this gives a measure of the spontaneous and total IRE-BP activity, respectively. In control T lymphocytes, total IRE-BP activity was unmodified during T lymphocyte activation whereas the spontaneous activity progressively rose starting from day 1 and peaked on day 3 of culture (Fig. 2). It is of interest that the increase in free IRE-BP activity directly and strictly correlates with the kinetics of TfR RNA expression. In iron-treated cultures the total activity of IRE-BP remained constant during the activation of T lymphocytes and was comparable to that observed in control dishes. In these cultures, the spontaneous IRE-BP activity did not increase during T lymphocyte activation (Figs. 1 and 2); thus, at day 3 of culture, the spontaneous IRE-BP activity corresponded to 11% of the total IRE-BP activity whereas in control cultures without iron salts, free IRE-BP activity rose to 33% of the total IRE-BP activity (Fig. 2). This finding is in agreement with the markedly lower expression of TfR RNA in iron-supplemented lymphocyte cultures as compared with controls.

Ferritin Expression in Mitogen-activated T Lymphocytes—In parallel we evaluated the ferritin content and the level of ferritin mRNA in PHA-stimulated T lymphocytes at different days of culture (Table I). The level of ferritin L-chain mRNA

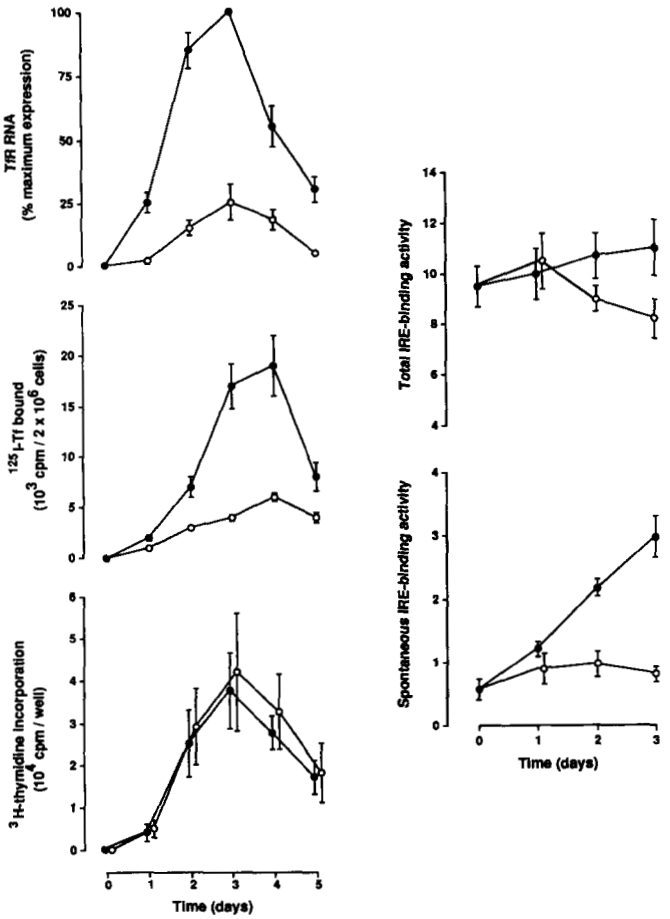

FIG. 1. **[^{3}H]Thymidine incorporation, ^{125}I-transferrin (*Tf*) binding capacity, TfR RNA expression, and IRE-BP activity in cellular extracts derived from PHA-stimulated human T lymphocytes grown in the absence (control, ●) or presence (○) of ferric ammonium citrate (mean ± S.E. from three separate experiments).** Human T lymphocytes were grown in the presence of PHA (5 μg/ml) and in the absence or presence of ferric ammonium citrate (25 μg/ml). [^{3}H]Thymidine incorporation, ^{125}I-transferrin binding capacity, and TfR RNA level were evaluated at each day of culture as described under "Experimental Procedures." For TfR RNA expression 10 μg of total RNA was run on 1% formaldehyde-agarose gels, transferred onto nitrocellulose paper, and hybridized to the human TfR cDNA probe (pcD-TR1). The blots were hybridized with the β-actin probe for normalization of RNA level. TfR RNA levels were then evaluated after scanning the autoradiograms and expressed as percentage of maximum levels. The level of IRE-BP activity was evaluated by quantitation by laser densitometry of the RNA-protein complex from autoradiographs similar to those shown in Fig. 2 (mean ± S.E. from three separate experiments).

decreased progressively during T lymphocyte activation; the H-chain mRNA level increased only slightly. Furthermore, the addition of iron salts did not alter the level of L-chain mRNA significantly whereas it enhanced that of H-chain mRNA slightly. The quantification of ferritin content in T lymphocytes lysates showed that (i) in control T lymphocytes a progressive decrease of the ferritin content is observed during the different days of culture; (ii) in iron-supplemented T lymphocytes a very pronounced increase of the ferritin content is observed (Table I).

TfR Expression and IRE-BP Activity in Monocytes-Macrophages—To evaluate the effect of iron salts on the expression of TfR in maturing monocytes-macrophages, we added ferric ammonium citrate (50 μg/ml) to monocyte cultures and measured TfR expression at both protein (^{125}I-transferrin binding assay) and RNA (Northern blot analysis) levels at different

times thereafter. TfR were virtually undetectable on freshly isolated monocytes and were detected on cultured monocytes from day 3 onward. Monocytes grown in the presence of ferric ammonium citrate exhibited a transferrin binding capacity higher than that of controls (Fig. 3). Similar results were obtained when TfR expression was analyzed at RNA level. Thus, at days 3 and 6 of culture total RNA was extracted from monocytes grown in the absence or presence of ferric ammonium citrate (50 μg/ml). No TfR RNA was detected in control fresh monocytes. However, starting from day 1 of culture the monocytes showed a gradual rise of the 5.1-kb TfR transcript level up to peak expression on day 6 (Fig. 3). Iron-supplemented monocytes exhibited higher levels of TfR RNA, particularly at day 3 (Fig. 3).

Parallel experiments were performed to evaluate the activity of IRE-BP in cytoplasmic extracts from monocytes grown *in vitro* in either the absence or presence of ferric ammonium citrate. Results showed that the levels of spontaneous IRE-BP activity are moderately higher in iron-supplemented cultures than in controls, particularly at day 3 of culture (Fig. 4).

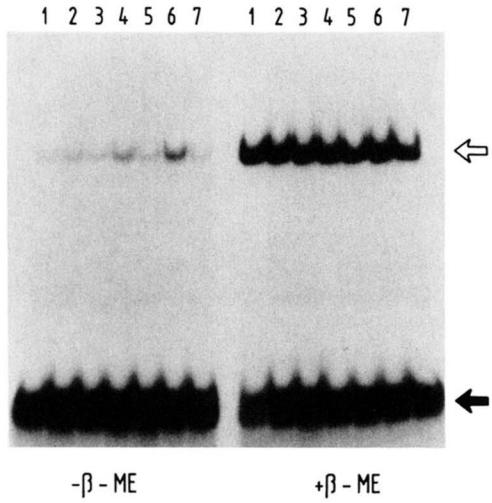

FIG. 2. **IRE-BP activity in cellular extracts derived from PHA-stimulated human T lymphocytes grown in the absence or presence of ferric ammonium citrate.** Human T lymphocytes, grown in cultures treated with PHA (5 μg/ml) in the absence or presence of ferric ammonium citrate (25 μg/ml) were then processed for the evaluation of IRE-BP content. Radiolabeled RNA (0.2 ng, 1.3 × 10^5 cpm) transcribed *in vitro* from pSPT-fer, containing the 5′ IRE of human ferritin heavy chain mRNA, was incubated at room temperature with 1-μg aliquots of protein cytoplasmic extracts. The extracts were derived from freshly isolated T lymphocytes (*lane 1*); T cells grown in the presence of PHA for 1 (*lane 2*), 2 (*lane 4*), and 3 days (*lane 6*); and T lymphocytes grown for 1 (*lane 3*), 2 (*lane 5*), and 3 days (*lane 7*) in the presence of PHA and ferric ammonium citrate (25 μg/ml). After sequential addition of RNase T1 and heparin, RNA-protein complexes (*open arrow*) were analyzed in 4% nondenaturing polyacrylamide gel. The incubation of cytoplasmic extract with the pSPT-fer transcript was performed after addition of 2% BME (+β-*ME*) or under standard conditions (−β-*ME*).

The quantitation of both spontaneous and total IRE-BP activity, performed by laser densitometry, showed that in freshly isolated monocytes, the spontaneous/total ratio of IRE-BP activity was 5% and rose to 28 and 27% at days 3 and 6 of culture, respectively (Figs. 3 and 4). In monocyte cultures supplemented with iron salts the spontaneous/total ratio is 40 and 28% at days 3 and 6 of culture, respectively (Figs. 3 and 4). An increase in the spontaneous IRE-BP activity was observed at day 3 of culture in iron-supplemented monocyte cultures as compared with that of corresponding controls, thus in agreement with data observed at the TfR RNA level. Finally, the level of total IRE-BP activity markedly and similarly increased in both control and iron-supplemented cultures from days 0 to 3 of culture (Figs. 3 and 4).

Ferritin Expression in Monocytes-Macrophages—Ferritin content and ferritin H- and L-chain mRNA levels were evaluated in monocytes maturing *in vitro* to macrophages in either the absence or the presence of iron salts (Table II). In control monocytes we observed at day 3 and 6 of culture pronounced rise of both ferritin H- and L- chain mRNA levels as well of the ferritin content. In iron-supplemented cultures the ferritin mRNA levels are only sligtly higher than those observed in control monocytes whereas the ferritin content is significantly more elevated than in controls (Table II).

DISCUSSION

Preliminary studies, based on either gel shift or ultraviolet cross-linking analysis, demonstrated that the IRE-BP activity increased in lysates of iron-starved cells and decreased in iron-loaded ones (16, 17). Further studies showed that the cytosol contains IRE-BP with two distinct affinities: one with a dissociation constant (K_d) of 10–30 pM, and the other with a K_d of 2–5 nM (26). The biochemical basis for the affinity change of the IRE-BP is apparently represented by the reversible oxidation-reduction of one or more sulfhydryl groups in the protein (30); the IRE-BP is in the oxidized form in cells that have been maximally activated by treatment with an iron chelator whereas in cells treated with iron virtually all the IRE-BP is in a reduced form (26, 30). It has been shown recently that IRE-BP activity is modulated by the binding of either protoporphyrin IX or hemin (27); thus, IRE-BP bound to protoporphyrin IX is in its high affinity state whereas when it binds heme it is in its low affinity state (27). The intracellular iron concentration determines the proportion of intracellular protoporphyrin IX with respect to heme.

This study aimed to analyze the regulation of IRE-BP activity by iron in cell types in which TfR expression is

TABLE I

Ferritin content and mRNA expression in PHA-activated human T lymphocytes

Ferritin content and ferritin mRNA expression were evaluated in PHA-activated human T lymphocytes by a radioimmunoassay and Northern blot, respectively. The data represent the mean ± S.E. values from three separate experiments.

Day	Ferritin content		Ferritin mRNA			
	Control	Ferric ammonium citrate (25 μg/ml)	Control		Ferric ammonium citrate	
			H chain	L chain	H chain	L chain
	ng/mg protein		*% day 0 expression*			
0	175 ± 55	175 ± 55	100	100	100	100
1	128 ± 27	345 ± 96	104 ± 8	83 ± 4	242 ± 29	88 ± 12
2	70 ± 19	707 ± 104	102 ± 12	66 ± 10	258 ± 45	78 ± 19
3	44 ± 22	598 ± 81	141 ± 7	46 ± 11	105 ± 12	36 ± 8

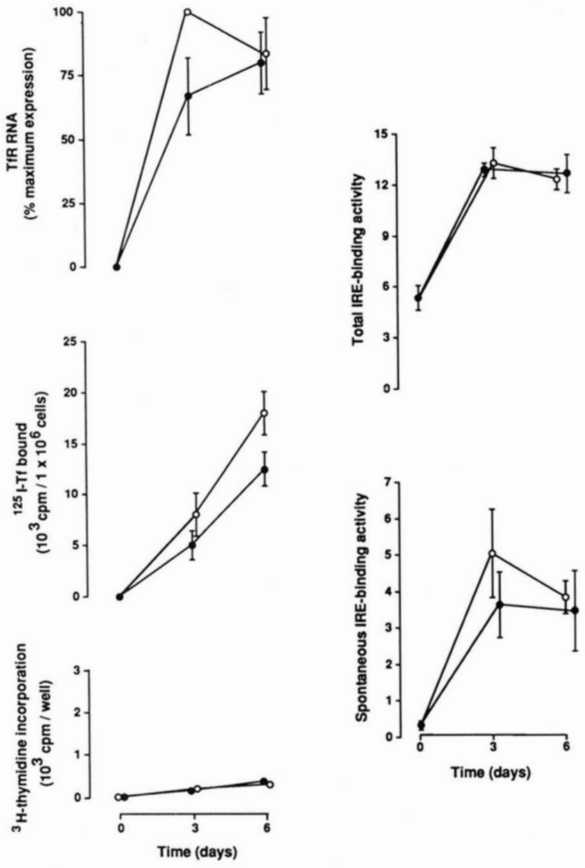

FIG. 3. **^{125}I-Transferrin (*Tf*) binding capacity, TfR RNA expression, and IRE-BP activity in cellular extracts derived from blood monocytes grown in the absence (control, ●) or presence (○) of ferric ammonium citrate (50 μg/ml).** ^{125}I-Transferrin binding capacity, TfR RNA expression, and [^{3}H]thymidine incorporation were evaluated as described in the legend to Fig. 1 (mean ± S.E. from three separate experiments). The level of IRE-BP activity was evaluated by quantitation by laser densitometry of the RNA-protein complex from autoradiographs similar to those shown in Fig. 4 (mean ± S.E. from three separate experiments).

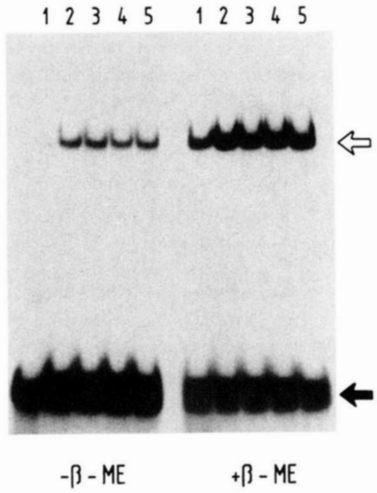

FIG. 4. **IRE-BP activity in cellular extracts derived from monocytes grown in the absence or presence of ferric ammonium citrate and then processed for the evaluation of IRE-BP activity.** This analysis was performed as described in the legend to Fig. 2. The cellular extracts were derived from freshly isolated monocytes (*lane 1*), monocytes grown in the absence of iron for 3 (*lane 2*) or 6 days (*lane 4*), and monocytes grown in the presence of iron salts for 3 (*lane 3*) or 6 days (*lane 5*). The procedure was performed after the addition of 2% BME (+β-*ME*) or under standard conditions (−β-*ME*).

differentially modulated by this element. Previous studies have shown that the intracellular iron level exerts a negative feedback on TfR expression in cells requiring iron for their proliferation, in contrast to the positive feedback observed in cells physiologically involved in iron storage, *i.e.* monocytes-macrophages (7, 11).

The experiments performed in PHA-stimulated T lymphocytes showed that in quiescent T lymphocytes, which do not express TfR but actively synthesize ferritin chains, only a small fraction of IRE-BP is present in its high affinity state. After T lymphocyte activation, the IRE-BP is progressively activated, as shown by the gradual increase in the spontaneous activity of IRE-BP, whereas the total IRE-BP activity remains unmodified. The addition of iron to PHA-stimulated T lymphocytes cultures elicited a complete inhibition of spontaneous IRE-BP activity as compared with the values observed in control lymphocyte cultures. In this regard, two aspects are of interest. (i) Spontaneous IRE-BP activity, virtually absent in quiescent T lymphocytes, is induced in activated T cells in parallel with the rise of TfR expression and decrease of ferritin content; this correlation suggests that activation of IRE-BP represents a key molecular mechanism underlying TfR and ferritin expression during the mitogenic activation of T lymphocytes. (ii) In mitogen-activated T cells an iron load down-modulates both TfR expression and spontaneous IRE-BP activity whereas it markedly enhances ferritin accumulation. Altogether these observations are in line with a two-step model previously proposed for TfR expression during T lymphocyte activation (7). This model essentially suggests that (i) in resting T cells, mitogen stimulation renders the TfR gene transcriptionally active; (ii) in activated T lymphocytes the depletion of the iron (or heme) intracellular pool leads to an induction of TfR RNA expression and to a reduction of ferritin synthesis (7). The results of the present study suggest that the depletion of the intracellular iron pool both stimulates TfR expression and inhibits ferritin synthesis via activation of IRE-BP. Our data do not exclude that growth stimulation acts in addition by a second mechanism, which permits TfR mRNA to be expressed in the cytoplasm, where its stability is modulated by IRE-BP. In fact, in the presence of iron salts, TfR mRNA increases to some extent although IRE binding activity remains the same (Figs. 1 and 2). A two-step model of TfR mRNA regulation would also be compatible with previous data showing that (i) iron chelation is not sufficient to induce TfR mRNA in quiescent T cells (7) and (ii) a deletion of the 3′-regulatory region in TfR mRNA did not entirely abolish the growth-dependent control in arrested NIH-3T3 cells (40).

Both spontaneous and total IRE-BP activity show an increase in monocytes maturing to macrophages *in vitro*. Here again, the spontaneous IRE-BP activity is very low in freshly isolated monocytes that do not express TfR and already synthesize ferritin but becomes markedly enhanced in monocytes grown for 3–6 days in culture, when TfR expression is clearly observed and ferritin expression is markedly enhanced at both mRNA and protein level. The addition of iron salts in monocyte cultures elicited a moderate stimulation of spontaneous IRE-BP activity particularly at day 3 of culture (in contrast with the inhibition observed for PHA-activated T lymphocytes) whereas total IRE-BP activity was comparable to that observed in the controls. This finding correlates with our previous observation that iron up-modulates TfR expression in monocytes maturing to macrophages (11). More importantly, these results lend further support to the conclusion that spontaneous IRE-BP activity plays a key role in the

TABLE II

Ferritin content and mRNA expression in human monocyte culture

Ferritin content and ferritin mRNA expression were evaluated in monocyte culture by a radioimmunoassay and Northern blot, respectively. The data represent the mean ± S.E. values from three experiments.

Day	Ferritin content		Ferritin mRNA			
	Control	Ferric ammonium citrate (50 μg/ml)	Control		Ferric ammonium citrate	
			H chain	L chain	H chain	L chain
	ng/mg protein		*% day 0 expression*			
0	445 ± 74	445 ± 74	100	100	100	100
1	802 ± 69	4450 ± 795	255 ± 32	220 ± 18	320 ± 51	310 ± 55
3	1015 ± 144	6650 ± 1420	1060 ± 84	1540 ± 77	1400 ± 94	2500 ± 209
6	2079 ± 214	4955 ± 440	790 ± 102	1330 ± 104	940 ± 79	1180 ± 88

modulation of TfR expression. It must be emphasized, however, that in monocytes maturing to macrophages the ferritin level shows an increase rather than a decrease, particularly after an iron load (11). Further studies are thus necessary to clarify the apparent dichotomy of the effects of IRE-BP in monocytes-macrophages, *i.e.* the rise of the spontaneous IRE-BP activity is linked not only to a rise of TfR expression at the RNA and protein level but also to ferritin expression at both mRNA and protein level. It is conceivable that the remarkable increase in ferritin genes expression observed during maturation of monocytes to macrophages may be related to molecular mechanisms other than IRE-BP. Similarly, the stimulation of ferritin synthesis elicited in these cells by iron load cannot be accounted for by IRE-BP.

In conclusion, the results of the present study shed light on the mechanisms responsible for the regulation of TfR gene expression. We suggest that the activated IRE-BP is directly involved in the induction of TfR gene expression in both a cell proliferation model (PHA-stimulated T cells) and a cell differentiation model (monocytes maturing to macrophages). In both models the spontaneous IRE-BP activity parallels TfR expression. Furthermore, the levels of both spontaneous IRE-BP activity and TfR expression are differentially modulated by iron in lymphocytes and monocytes-macrophages, which utilize iron to sustain their proliferation and are involved in iron storage, respectively.

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