Transferrin-Independent Iron Uptake Supports B Lymphocyte Growth

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Raji, a malignant B-lymphocyte cell line containing Epstein-Barr virus genomic elements, has been conditioned to proliferate optimally in transferrin (Tf)-free medium containing a very low concentration of an iron salt. We provide evidence that an Tf-independent iron uptake system is physiologically important for maintaining the growth of these cells. The data show that Raji cells take up iron from iron salts using a relatively high-capacity, low-affinity, temperature- and calcium-dependent uptake system. The apparent capacity of this system increases when: (1) cells are cultured in Tf-free medium containing high concentrations of iron salt as opposed to medium containing Tf; and (2) when the iron salt concentration of Tf-free medium is lowered to about 1.6 μmol/L. Cellular iron uptake also increases when a maximum number of cells are in S and G2 and M cell phases of the cell cycle. The cells are sensitive to growth inhibition by the addition of deferoxamine. This evidence supports the hypothesis that certain malignant lymphocytes, under iron deplete conditions, fulfill an iron requirement for proliferation by an adaptation such as Tf-independent iron uptake.

IRON (Fe) IS A UNIVERSAL requirement for cellular proliferation, particularly for rapidly growing tumor cells. Under usual conditions, increased Fe is provided to proliferating cells by an increase in transferrin (Tf) receptor expression. This increased expression allows for increased uptake of the plasma Fe transport protein, Tf, so that more Fe can be available for essential cellular processes. At least one of these processes is related to an Fe requirement for DNA synthesis.

Because both normal and malignant lymphocytes exhibit a wide range of proliferative responses, it would not be surprising to find additional adaptations that would increase use of agents such as Fe. In one such important adaptation, Tf synthesis by T4 (helper) lymphocytes appears to facilitate T-lymphocyte proliferation. Teloigically, this adaptation may result from the fact that areas of the lymph node are poorly vascularized, and these cells cannot obtain enough Tf from serum. Other reports suggest that some malignant lymphocyte lines synthesized “Tf-like” factors that allow for continued proliferation in vitro.

Our studies have shown that Tf synthesis by certain malignant cells has important implications because synthesis by small cell lung cancer cells regulates autocrine control of tumor cell proliferation.

In view of the importance of increased Fe use by proliferating malignant lymphocytes, the use of agents that specifically inhibit Fe use, such as Fe chelators or gallium (Ga) nitrate, have been considered or even used in the treatment of high grade lymphomas and other malignancies.

In this report, we provide evidence that a malignant B-cell line uses a specific Tf-independent Fe uptake system that is permissive for Fe uptake from Fe salts. These malignant B cells do not show evidence for Tf synthesis, an adaptation demonstrated in a T-cell lymphoma line. Previous reports of Tf-independent Fe uptake have been described on perfused liver tissue and mouse leukemia cell lines. Most recently, this type of transport system has been extensively studied in HeLa cells. We provide evidence that such a system is physiologically important for maintaining growth of malignant B cells in medium containing neither added Tf nor high concentrations of Fe salts (low Fe medium).

MATERIALS AND METHODS

Cell Systems Used

Raji cells (derived from Burkitt’s lymphoma originally obtained from American Type Tissue Culture [ATTC], Rockville, MD) were kept as “stock” cultures in RPMI 1640 medium with 5% fetal calf serum (FCS) at 37°C in 7.5% CO₂ atmosphere. RPMI 1640 (M.A. Bioproducts, Walkersville, MD) with the addition of 200 μmol/L ethanolamine, 50 μmol/L hydrocortisone, and 200 μmol/L ascorbic acid, is the basic protein-free medium used for serum-free conditions (adapted from Vostrejs et al and Kovar). Cells were cultured in three different forms of serum-free media based on the following further additions to the RPMI: (1) 5 μg/mL of human Tf-Fe (Tf medium); (2) ferric citrate containing 500 μmol/L Fe (Fe salt medium); or (3) 1 μmol/L Fe-citrate (low Fe medium). This small amount of Fe is added so as to better “standardize” the amount of Fe in low Fe medium because, based on atomic absorption analysis, we have found that different batches of media with no addition contain from 0.2 to 1.2 μmol/L Fe (unpublished data).

We and others have shown that certain “Tf-free” medium or buffer may be contaminated with trace amounts of Tf. Using our previously described methodology we determined that the low Fe medium contained less than 1 ng of contaminating Tf per milliliter.

Measurements of cellular proliferation. For most experiments, cellular proliferation was determined by performing manual cell counts. In the studies with varying concentrations of deferoxamine, the number of living cells was determined by the photometric method of Mossman. Microtiter plates were read as previously described using a Titertek Multiskan instrument (model 2550 ELA reader; BioRad, Richmond, CA). To assess cell viability, manual cell counts were also performed on at least one well from
each row in the microtiter plate. Cell cycle measurements used propidium iodide staining of cells for DNA followed by analysis using a fluorescent-activated cell sorter as previously described. Determinations of Tf, ferritin, Tf receptor, and Fe. Tf, ferritin, and radioactivity associated with ferritin were assayed using immunoassays previously described. Expression of surface Tf receptors was assessed by performing [125I]-Tf binding studies as previously described. In some instances, “contaminating” Tf was eluted from cells before performing the assay (see below). Quantitation of surface Tf receptors by measuring maximal Tf binding used calculations we have described previously. Fe determinations on cell pellets or medium used a flameless atomic absorption method we have recently used. Fe based on the estimated inhibition constant for this uptake system (see below). All cells were then washed extensively and reconstituted in RPMI 1640 medium with no additions. For most uptake studies (unless otherwise stated), 4 x 10⁶ cells were incubated in 300 µL of RPMI 1640 medium with 50 µL of ³⁵Fe-citrate (to make a final concentration of 0.9 µmol/L ³⁵Fe) in 10 mmol/L Tris, pH 8.0, and 150 mmol/L NaCl. An additional 50 µL of this Tris buffer is also added because this buffer contained additives to the assay (see below). The ³⁵Fe-citrate was freshly made the day of each experiment because the preparation exhibits decreased solubility if kept at 4°C for more than 24 hours. Any additions to the assay were contained in the additional 50 µL of Tris-HCl, pH 8.0, 150 mmol/L NaCl. After the desired incubation period, 2 mL of ice-cold RPMI 1640 medium was added and the cells centrifuged at 500g, the supernatant aspirated, and then both cell pellet and supernatant were counted for radioactivity. In all experiments, it was important to perform the following controls to account for “nonspecific” uptake: (1) tubes containing cells in radioactive Fe were incubated in ice water; (2) tubes not containing cells were incubated with radioactive Fe and any of the additions at 37°C. In all instances, “control pellets” should contain 10% or less of the total radioactivity added. For uptake studies in tissue culture flasks, enough warm low Fe medium was added to cells in the low Fe medium so that the cell count in the various flasks was similar (around 0.4 x 10⁶/cells/mL) and enough ³⁵Fe-citrate added to make a 0.2 µmol/L concentration. After a 2-hour incubation period, cells were removed from the flasks, washed twice by centrifugation in ice-cold RPMI 1640 medium, and the pellet counted for radioactivity.

RESULTS

The low Fe medium used in these studies had 1 µmol/L Fe-citrate added and contained a total of 1.6 µmol/L Fe as measured by atomic absorption (see above). In previous experiments performed by us and others measuring cell proliferation using Tf-free medium with added Fe salts, it has been noted that, unless the cells synthesize Tf or the medium has Tf contamination, the cells proliferate poorly, if at all, in medium containing less than 10 µmol/L Fe. We previously determined that Raji cells removed from FCS grow equally well in Tf medium or Fe salt medium; 2 x 10⁶ cells/mL reach a concentration of 1 x 10⁶ cells at 6 days postculture. When Raji cells are taken from Fe salt medium and reconstituted in low Fe medium, cell proliferation is relatively slow because 5 x 10⁶ cells/mL double in number by 6 days. (Cells transferred from Tf medium to low Fe medium grow even slower, i.e., 8 to 10 days for cells to reach 1 x 10⁶/mL.) However, in all instances, by the third passage in low Fe medium, Raji cells proliferate at the same rate as cells in Tf or Fe salt medium. Raji cells grown in Fe salt medium contain about 10- to 50-fold higher cell-associated Fe as cells taken from either Tf medium or cells subcultured in Tf-free low Fe medium (data not shown). Although the amount of ferritin-associated Fe may vary considerably, the immunoreactive ferritin concentration directly correlates with cell-associated Fe. As shown in Table 1, cells grown in high Fe salt medium contain twice as much immunoreactive ferritin as cells in Tf medium and four times more than cells in low Fe medium. The majority of intracellular Fe is ferritin Fe, but much of the Fe associated with ferritin may not be readily available for cellular processes because (1) Tf-dependent cell lines exhibit growth arrest when Tf is removed from media despite adequate Fe stores in ferritin and (2) we have shown that cells grown in high Fe salt medium are extremely sensitive to inhibition of cellular proliferation by Tf-Ga, an agent that interferes with Fe use. Although ferritin Fe is not readily available for cellular reactions such as proliferation, ferritin synthesis may increase rapidly when less Fe is used for the cellular reactions and there is an increase in the intracellular “chelatable” Fe pool. Measurements of immunoreactive ferritin have been shown to be extremely sensitive to rapid changes in Fe requirements, and, therefore, ferritin decreases during active cellular proliferation in vitro in medium containing Tf. As shown in Table 1, all three cell lines exhibit significant decreases in cellular ferritin content when cells

| Table 1. Measurements Performed on Raji Cells in Different Media |
|-----------------|-----------------|-----------------|
| **Media**       | **Log Phase**   | **Ferritin**    |
|                 | Growth*         | (ng/10⁶ cells)† | **Maximal Tf**  |
|                 |                 |                  | Binding†        |
| Fe-citrate (500 µmol/L iron as added Fe-citrate) | Yes | 28.6 ± 4.2 | 5.9 ± 0.4 |
|                 | No             | 38.4 ± 3.6      | 5.8 ± 0.5       |
| TF-Fe (0.6 µmol/L iron in RPMI and 0.125 µmol/L bound to Tf) | Yes | 12.6 ± 2.4 | 13.4 ± 1.2 |
|                 | No             | 21.4 ± 3.0      | 5.2 ± 0.4       |
| Low iron (0.6 µmol/L iron and 1.0 µmol/L as added Fe-citrate) | Yes | 4.2 ± 0.8 | 7.0 ± 0.7 |
|                 | No             | 9.6 ± 1.3       | 7.2 ± 0.4       |

*Log phase cells were those taken at the beginning of day 3 postsubculture with 50% to 100% more cells in S and G2 and M phases of the cell cycle as compared with cells not in log phase taken at the beginning of day 6 when cell density is highest.
†Mean ± SD of three determinations.
are in "log phase" of growth. This change strongly supports the hypothesis that all three sets of cells, including those in low Fe medium, use and require Fe for maintenance of proliferation.

Also shown in Table 1, surface Tf receptors measured by [125I]-binding are higher in cells grown in low Fe medium as compared with cells grown in Fe salt medium. However, Tf receptor does not increase during increased proliferation for both cells grown in Fe salt medium as well as low Fe medium. Thus, there is no increase in [125I]-Tf binding at the beginning of the day 3 (Table 1) as well as at the beginning of the day 4 postsubculture (data not shown) when compared with cells taken just before subculture. [125I]-Tf binding sites do increase for cells grown in Tf medium (Table 1), where Tf-mediated Fe uptake is essential for proliferation.5

Because Raji cells did not require Tf-mediated Fe uptake for growth, assays were performed to measure uptake of Fe from Fe salts (Fe-citrate). The assays were performed in RPMI 1640 medium with 0.9 μmol/L added 59Fe-citrate so as to best reproduce culture conditions when cells are in low Fe medium. In Fig 1A, uptake of 59Fe by cells removed from the various media is measured at different timepoints. Under the assay conditions, uptake at 37°C occurs in a linear fashion for cells removed from all forms of media for the first 20 to 30 minutes, at which time the curve becomes less steep. As noted in Materials and Methods, the assay mixture containing various cells has neither significant contamination with Tf, nor a significant excess of nonradioactive Fe salts (see above and Fig 1 legend). For all three sets of cells when incubated with 59Fe for 2 hours, greater than 60% of the cell-associated radioactive Fe was associated with ferritin, providing indirect evidence that the incorporated radioactive Fe was being effectively used by the cells (data not shown).

Although all three sets of cells show significant uptake of Fe from the added 59Fe-citrate (Fig 1A), cells taken from Fe salt medium have significantly higher uptake at all timepoints as compared with cells taken from Tf-Fe-containing medium. This finding supports studies of Tf-independent Fe uptake as described in other cells that indicate that the capacity of this system is increased when the Fe salt concentration of the medium is increased (Dr J. Kaplan, personal communication, November 1990). More importantly, in the present studies, we also show that Raji cells maintained in low Fe medium show an even greater and significant increase in uptake as compared with cells grown in high Fe salt concentration.

The rate of Fe uptake exhibited by all three sets of cells shown in Fig 1A suggests that, under the assay conditions, uptake appears to approach saturation in all that the curves become less steep between 30 and 60 minutes. To determine if the system is saturable as well as to estimate relative affinity, the 30-minute timepoint was chosen to measure the effects of the addition of increasing amounts of nonradioactive Fe salts. Under the assay conditions, all three sets of cells exhibit inhibition of Fe uptake, and in each case, about a 60-fold excess of nonradioactive Fe salt results in 50% inhibition of uptake, indicative of similar inhibition constants (Ki). This result strongly indicates that all three sets of cells use the same uptake system. Based on the estimated Kis shown in Fig 1B and the other data shown, it can be concluded that Raji cells have a saturable, low-affinity, and based on this low affinity) relatively high-capacity Fe uptake system that is more permissive at 37°C when

![Fig 1](https://www.bloodjournal.org/journals/doi/fig/1)

(A) Uptake of 59Fe by Raji cells taken from various media as described in Materials and Methods. Cells were all at 5 days postsubculture and had similar cell density. For these experiments, the 0.9 μmol/L 59Fe-citrate was added to 4 x 10⁷ cells in RPMI 1640 medium with no additions, and uptake was measured at various time points at 37°C. All uptake experiments, therefore, were performed in medium almost identical to low iron medium. The concentration of nonradioactive iron in the RPMI 1640 medium was 0.6 μmol/L, and cells taken from low iron medium increased this nonradioactive iron concentration an additional 0.2 μmol/L, whereas the other cells added did not significantly increase the total iron concentration of the assay (see Materials and Methods). The points at the y axis represent uptake measured at 4°C. The bars represent ±SD for three determinations. (B) Cells taken from low Fe medium; (○) cells from Fe salt medium; (●) cells from Tf medium. (B) Under the same conditions as shown in (A), uptake from added 59Fe-citrate is measured at 30 minutes at 37°C in the presence of increasing molar excess of added nonradioactive Fe-citrate. The points seen at the y axis indicate uptake without added nonradioactive Fe-citrate. Points represent a mean of two determinations.
compared with results at 4°C. When Fe uptake is measured in medium containing an Fe concentration similar to that in the low Fe medium, the apparent capacity of the uptake system increases for (1) cells continuously cultured in medium with Fe salts and no added Tf, and (2) increases further for Raji cells that have been continuously cultured in low Fe medium.

A previous study of HeLa cells indicated that ⁵⁹Fe uptake was calcium dependent. We found that uptake in calcium-citrate.

The data shown in Table 2 list specific agents that affect ⁵⁹Fe uptake by cells grown in low Fe medium under assay conditions shown in Fig 1. Uptake is measured for 0.5 hour with preincubation of cells with certain agents. As noted in Table 2, other nonradioactive metal salts will inhibit ⁵⁹Fe uptake. For example, Ga and magnesium inhibit Fe uptake at concentrations similar to inhibition caused by added Fe salts (Table 1 and Fig 1B). Zinc results in less inhibition of uptake (data not shown).

Table 2 also shows that when the calcium content of RPMI 1640 medium is increased from the usual 0.4 mmol/L to 2 mmol/L uptake increases. Similarly, the addition of ionomycin, an agent that increases intracellular calcium, also results in a slight but significant increase in uptake (P < .03 as compared with no addition; Table 2).

Uptake of Fe is inhibited if calcium uptake is inhibited by a calcium channel blocker. Thus, when verapamil is added to the uptake system, significant inhibition results (Table 2). These data also support the concept that the calcium dependence of this uptake system is at least, in part, related to calcium influx. It is known that B cells possess calcium channels that are inhibited only in the presence of relatively higher concentrations of calcium channel blockers than those required for cells with voltage-gated calcium channels. However, under the conditions of the assay even high concentrations of verapamil do not completely inhibit Fe uptake, suggesting that further studies will be necessary to determine the total dependence of the Fe uptake system on calcium influx as well on intracellular calcium concentration.

We next determined if the Fe uptake system was physiologically important for maintaining cell proliferation. In initial studies, the same numbers of cells taken from all three forms of serum-free media exhibited similar MTT values. This result indicates that cells grown in the low Fe medium have the ability to maintain oxidative mitochondrial phosphorylation, an Fe-requiring process that is indirectly measured in the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. When deferoxamine (DFO) is added to cells previously cultured for three passages in low Fe medium, this Fe chelator causes a dose-related inhibition of cellular proliferation. A DFO concentration of about 2 pg/mL results in 50% inhibition of proliferation occurs at about 2 μg/mL of deferoxamine. The same concentration of deferoxamine results in inhibition of proliferation when assessed by manual cell counts (data not shown).
The high concentration of DFO required to inhibit proliferation in high Fe salt medium undoubtedly reflects binding of the large excess of extracellular Fe to the DFO. Less DFO necessary to inhibit proliferation in low Fe medium as opposed to Tf-Fe medium may reflect either (1) more efficient chelation of extracellular Fe not bound to Tf\(^2\); or (2) a lower concentration of intracellular "chelatable" Fe present in cells cultured in the low Fe medium as compared with cells grown in Tf-Fe, as reflected by differences in cellular ferritin content shown in Table 1. However, whatever the explanation DFO inhibition of cellular growth strongly supports the hypotheses that (1) cells grown in low Fe medium require Fe for maintenance of cellular proliferation; and (2) the Fe is provided by a Tf-independent Fe uptake system.

The physiologic importance of the system was also assessed during cell growth. Uptake studies similar to those shown in Fig 1 performed on cells in log phase growth resulted in a modest but significant increase in Fe uptake as compared with cells obtained at the highest cell density. Uptake was then measured for 2 hours in the tissue culture flask, at which time the cells were in various stages of proliferation. In these experiments, a 0.2 \(\mu\)mol/L concentration of \(^{59}\)Fe-citrate was added to the cells in separate tissue culture flasks. Under actual tissue culture conditions, significantly increased \(^{59}\)Fe uptake was evident when a higher percent of cells had progressed through the cell cycle. Thus, by the beginning of day 3, when a maximum number of cells were in S and G2/M phase, there was a threefold increase in \(^{59}\)Fe uptake/10\(^6\) cells as compared with cells at day 1.

Using our previously described methodology that demonstrated Tf synthesis by small cell lung cancer cells,\(^1\) we found that Raji cells exhibit no detectable synthesis of Tf and have neither measurable amounts of cell-associated Tf (<0.2 ng/10\(^6\) cells) nor measurable Tf in conditioned medium (<2 ng/mL). Raji also did not exhibit Tf synthesis using our previously described methodology to measure specific \(^{35}\)S-methionine incorporation into Tf.\(^1\) The addition of a specific monoclonal antibody (MoAb) directed against the Tf receptor that inhibits proliferation of Tf dependent cell lines\(^2\) to Raji cells in low Fe medium does not inhibit proliferation.

We sought to determine if other lymphocyte lines could use the Tf-independent Fe uptake system to sustain growth. Of the seven other lymphocyte lines tested to date, only one line, HSCE\(^T\), appears to use this Tf-independent Fe uptake system to maintain optimal cellular proliferation in low Fe medium. HSCE\(^T\) cells, like Raji, contain Epstein-Barr virus (EBV) genomic elements.\(^8\) Only one of six EBV-negative cell lines (fr-e of B-cell lineage and one of T-cell lineage) was also able to sustain optimal growth in low Fe medium. This cell line, Jurkat, is a T-cell lymphoma line. Using our methodology described above,\(^1\) we found that Jurkat cells contain immunoreactive human Tf (2.4 ng/10\(^6\) cells). Jurkat cells also exhibit Tf synthesis as evidenced by specific \(^{35}\)S-methionine incorporation into Tf,\(^1\) and medium conditioned by Jurkat cells contains measurable Tf (about 20 ng Tf/mL). The addition of specific MoAb against Tf receptor\(^25\) inhibits the proliferation of Jurkat cells grown in low Fe medium, demonstrating the importance of the Tf receptor pathway for maintaining proliferation of Jurkat cells in this medium.\(^27\) Further definition of Tf synthesis by Jurkat cells is currently underway.

**DISCUSSION**

In this report, we show that Raji cells, a malignant B-cell line containing EBV genomic elements, take up Fe from Fe salts using a relatively high-capacity, low-affinity, temperature- and calcium-dependent uptake system. This system appears to be similar to another Tf-independent Fe uptake system shown to exist on normal liver cells\(^14\) and more recently described for HeLa cells.\(^16\) As with the system described in this report, the system described on HeLa cells also appears to be permissive for uptake of other metals.\(^15\) Thus, it seems plausible that under certain physiologic conditions this transport system is intended to transport another cation or cations that, under normal conditions, do not have carrier proteins.

The present studies agree that Tf-independent radioactive Fe uptake is increased under conditions that allow for increased cellular Fe uptake such as the addition of high concentrations of Fe salts to medium.\(^14,28\) Therefore, these findings support the hypothesis that a number of different cell types have the capacity to take up Fe, particularly when extracellular Fe concentrations are high.\(^14,28\) This latter adaptation suggests that the system may have functional importance in removing potentially toxic free Fe molecules under conditions of Fe overload.\(^28\)

More importantly, in the present studies we provide evidence that this Tf-independent Fe uptake system is physiologically important for maintaining the growth of Raji cells that have been conditioned to proliferate optimally in Tf-free medium containing a very low concentration of an Fe salt. As opposed to other studies of cell growth in serum-free media without added Tf or high concentrations of Fe salts, this medium contains neither significant Tf contamination, nor do the cells show any evidence for endogenous Tf synthesis. The high capacity of the Tf-independent uptake system in Raji cells is not only maintained but actually increases when the cells are continuously cultured in this low Fe medium. Fe uptake is also increased when a maximum number of cells are in S G2/M phases of the cell cycle. The cells are sensitive to growth inhibition by DFO, providing additional evidence that in this low Fe medium, Fe is still a requirement for these cells to maintain cell growth. Of seven B-cell lines studied to date, the only two that maintain growth by using the system are also the only ones that contain EBV genomic elements.

The description of this uptake system in a B-cell line and demonstration of specialized Tf synthesis by T cells (Jurkat) support the hypothesis that malignant lymphocytes have the capacity to respond to Fe depletion by adaptations that differ from one cell line to another and that these adaptations may be related to lymphocyte lineage. These different adaptations presumably allow for effective use of the small amounts of Fe salts available in the medium through either specific uptake of these Fe salts, or by Tf synthesis so that the Fe salts can be more effectively taken up through the Tf receptor pathway. These adaptations may be important in vivo for lymphocyte proliferation.
under conditions in which optimal amounts of Tf-bound Fe are not readily available. Although a large amount of Tf-bound Fe is normally present in plasma, concentrations of Tf-bound Fe in different extracellular fluids as well as the amount of available non-Tf-bound Fe are presently unknown. Specialized synthesis and secretion of endogenous Tf, by normal and malignant cells in vitro, as well as in vivo examples such as specialized Tf synthesis by Sertoli cells of the testes and oligodendrocytes in brain, presupposes that small amounts of non-Tf-bound Fe found in extracellular fluid must be made available for proliferating cells that exhibit this adaptation. This form of adaptation may be important for optimal growth of malignant lymphomas where large, rapidly proliferating tumors may not be able to obtain enough Tf from serum.

Further characterization of these uptake systems in defining growth regulation of both normal and malignant lymphocytes is underway. These studies may lead to novel therapeutic approaches, particularly for the treatment of lymphomas that may use these specific systems for maintenance of cell growth.

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