Specific Binding Sites for H-Ferritin on Human Lymphocytes: Modulation During Cellular Proliferation and Potential Implication in Cell Growth Control

By Silvia Fargion, Anna Ludovica Fracanzani, Bruno Brando, Paolo Arosio, Sonia Levi, and Gemino Fiorelli

FERRITIN, AN IRON-BINDING protein present in almost all living cells, is composed of 24 subunits of two types, known as H and L, which assemble in different proportions in the various tissues. The major function of ferritin is to store intracellular iron. The two subunits appear to have specialized roles: H-ferritin has a ferroxidase activity and is involved in rapid iron uptake and iron detoxification, whereas the more stable L-ferritin is involved in long-term iron storage.

Other functions unrelated to iron storage have been attributed to ferritin: the major function of ferritin is to store intracellular iron. The two subunits involved in long-term iron storage are H and L, which assemble in different proportions in the various tissues. The major function of ferritin is to store intracellular iron. The two subunits appear to have specialized roles: H-ferritin has a ferroxidase activity and is involved in rapid iron uptake and iron detoxification, whereas the more stable L-ferritin is involved in long-term iron storage.

Dual-color flow cytometry experiments showed that ferritin binding sites were present on cells expressing the proliferation markers membrane ferritin was mainly recognized by anti-H-ferritin antibodies. The origin and significance of lymphocyte-bound ferritin remained unclear.

Membrane binding sites specific for H-ferritin were identified in various cell lines such as K562 and HL60, and on erythroid progenitor cells, and their expression was shown to be modulated by cellular proliferation and differentiation. The existence of such binding sites on lymphocytes might explain both the presence of membrane ferritin and the inhibitory activity of ferritin on in vitro lymphocyte proliferation and migration.

We investigated in vitro binding of rHF and rLFo to lymphocytes in different stages of growth and the effects of such ferritins on lymphocyte proliferation. Our results show the presence of specific H-ferritin binding sites on proliferating lymphocytes, which are probably involved in the H-ferritin suppressive activity.

**MATERIALS AND METHODS**

**Lymphocytes.** Lymphocytes were isolated from peripheral blood (PB) of normal human donors by Ficoll-Hypaque gradient centrifugation. Cells (5 × 10⁶) were cultured for 72 hours in 0.6 mL RPMI 1640 (Flow Laboratories, Irvine, Scotland, UK) with addition of 10% FCS (Flow) with or without 10 μg (GIBCO, Grand Island, NY).

**Ferritins.** rHF and rLFo were over expressed in Escherichia coli, extracted, and purified as previously described. L-Ferritin carries a substitution of the first two amino acids Ser-Ser by Asp-Pro and is therefore named rLFo. Both proteins were pure by electrophoresis analyses.

**Iodination of ferritins.** Ferritins were freshly iodinated before each experiment, as described by Fraher and Speck. Forty micrograms purified rHF and rLFo was added to 1 mCi (1 μg) sodium iodide (Amersham, England) with 10 μg 1 mg/mL 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Iodo-gen, Sigma, St Louis, MO), and incubated for 20 minutes at 37°C. Bound radioactivity was separated by gel filtration on Sephadex G25 (Pharmacia, Uppsala, Sweden). The specific radioactivity was 7,000 to 15,000 cpm/mg for rHF and 2,500 to 5,000 cpm/mg for rLFo, 95% to 98% precipitable by 10% trichloroacetic acid (TCA).

**Ferritin binding.** Ferritin cell binding was performed as already described. Lymphocytes 2 × 10⁶ in 50 μL 10% fetal calf serum (FCS) RPMI 1640 were incubated for 60 minutes at 37°C with saturating concentrations of radioiodinated rHF or rLFo in 50 μL phosphate-buffered saline (PBS) containing 1 mg/mL bovine serum albumin (BSA). Cells were washed three times in saline, and the bound radioactivity was counted. Nonspecific binding was...
determined by addition of a 300-fold excess of unlabeled ferritins and subtracted from the total binding.

**Ferritin inhibitory activity.** Lymphocytes, $5 \times 10^7$ cells, were cultured as above for 72 hours in 0.6 mL, with or without addition of 10 $\mu$g PHA with various concentrations of ferritin. Methyl-3H-thymidine (2 $\mu$Ci) (Amersham) was added 4 hours before the end of the culture. Cells were harvested, and thymidine incorporation was assessed. The results were expressed as PHA index.20

**Fluorescence-activated cell sorter analysis (FACS).** To label the ferritin binding sites we used the following procedure: 10$^5$ lymphocytes suspended in 0.1 mL were incubated with 10 $\mu$L purified rHF or rLFo at 1 mg/mL for 30 minutes at 4°C, washed three times in 3 mL PBS supplemented with 0.1% sodium azide and 2% inactivated FCS pH 7.2, incubated for 40 minutes with 50 $\mu$L 0.5 mg/mL monoclonal antibodies (MoAbs) 2A4 and LF03 specific for the H- and L-ferritins, respectively,20,26 washed again, and incubated with 8 $\mu$L FITC-labeled goat anti-mouse immunoglobulin (GAM-Ig) antibody (Becton Dickinson, Mountain View, CA) for 25 minutes at 4°C. Cells were washed again and analyzed by flow cytometry.27 Controls consisted of cells treated as above but without incubation with the antiferritin antibodies, which were replaced by a nonspecific mouse MoAb of the same isotype as the antiferritin antibody.

**Dual-color surface immunofluorescence.** The washed cells (50 $\mu$L), labeled as described above, were further incubated with 10 $\mu$L unrelated MoAb for 15 minutes at 4°C to saturate free residual binding sites. After the cells were washed, they were incubated with 25 $\mu$L phycoerythrin (PE)-conjugated MoAb for CD4 (Leu3a), CD8 (Leu2a), CD19 (Leu12), HLA-DR, MLR3 (CD69), IL-2-R (CD25), and Tf-R (CD71) (Becton Dickinson) for 20 minutes at 4°C. The cells were washed twice before flow cytometry analysis. Cell viability was estimated by addition of 50 $\mu$L 5 $\mu$g/mL ethidium bromide (Sigma) to 50 $\mu$L cell suspensions and analysis by flow cytometry.27,28 Only samples with viability greater than 90% were taken into consideration.

**DNA analysis in single and dual-color flow cytometry.** Cell membranes were made permeable by incubation in ice-cold 70% ethanol in PBS for 10 minutes, and DNA was then stained by incubation in 1 $\mu$g/mL propidium iodide (Sigma) for at least 3 hours in the dark. The cell cycle was calculated by analyzing the DNA of 50,000 cells in flow cytometry and computing the results with a sum of broadened rectangle algorithm (Becton Dickinson); the cycle phases were expressed as percentage values.29

**Dual-color DNA surface analysis.** DNA staining was performed on 0.2 to 0.5 mL cells previously labeled for surface ferritin as described above. The permeabilization procedure in 70% ethanol did not affect surface staining significantly.

**Flow cytometry.** A standard four-parameter flow cytometer, equipped with a 2-W argon-ion laser (FACS, Becton Dickinson) was used. For single- and dual-color surface analysis, the cells were gated with a forward v side scatter cytogram to exclude dead cells or aggregates. For dual-color analyses, an appropriate compensation network was set for FITC/PE combination. A forward/side scatter cytogram was also used to select cells for viability analysis. At least 10,000 cells were analyzed in every run. Fluorescence was detected in log amplification mode, and the results were expressed either as monoparametric histograms or as bivariate cytograms or contour plots. Negative control samples were used to set appropriate positive windows, and the background count in the control was always subtracted from the positive cell samples. For single- and dual-color DNA analysis, cells were selected on the basis of red (DNA) fluorescence with an appropriate threshold and trigger.

**Time-course studies.** Cells cultured in 10% FCS RPMI 1640 with or without PHA were harvested after 10-, 34-, 60-, and 84-hour culture. At each time, dual-color analysis was performed as described by Brando et al.28

**RESULTS**

**H- and L-ferritin binding sites.** Radiolabeled recombinant H- and L-ferritins were incubated with lymphocytes either untreated or incubated with or without PHA. L-ferritin binding was never detectable; in contrast, H-ferritin showed a significant binding to cultured lymphocytes, which increased more than threefold in the PHA-stimulated cells. Fresh, uncultured cells did not bind detectable ferritin (Table 1). Saturation experiments with increasing concentrations of $^{35}$I-rHF detected about 12,000 rHF binding sites in the PHA-activated cells, with an apparent $K_a$ of 1.4 $\times 10^5$ mol/L$^{-1}$ (data not shown). Parallel experiments with immunofluorescence flow cytometry detected a specific binding of L-ferritin to 2% of the cells or less, whereas H-ferritin bound 4% and 27% of the PHA-unstimulated and PHA-stimulated cells, respectively (Table 1).

Further flow cytometry experiments, aimed at assessing whether the low binding of L-ferritin was significant, showed background bindings of 3% to 5% positive cells with antiferritin antibodies alone or with mismatched antibodies and that rLFo (and its antibody) was not incremental to the fluorescence associated with rHF and its antibody (ranges of three experiments: 23% to 27.8% and 23.7% to 28.4%, respectively). Thus, the present approach does not demonstrate specific binding sites for the L-ferritin on lymphocytes. Further binding studies were performed with H-ferritin.

**H-ferritin binding in relation to lymphocyte markers.** PHA-stimulated cells were labeled for rHF binding with green FITC antibodies and for other lymphocyte markers with PE-conjugated antibodies. Dual-color flow cytometry results are shown in Fig 1 and summarized in Table 2. H-Ft-BS were found on all CD19$^+$ B cells, on about 30% of CD4$^+$ T-helper cells, and on 30% of CD8$^+$ T-suppressor subsets (FigIA). All H-Ft-BS$^+$ cells expressed the HLA-DR, MLR3, and Tf-R antigens, and a major proportion of H-Ft-BS$^+$ cells (67%) were IL-2-R$^+$ + . H-Ft-BS and Tf-R (ie, the two iron-binding protein receptors) were proportionately expressed, which was not so evident with the other activation markers (Fig IB).

We used flow cytometry to analyze the time course of the expression of H-Ft-BS and the other activation markers during PHA stimulation (Fig 2). All the markers tended to increase in a parallel fashion and reached a maximum after 60-hour incubation, as previously reported.28 Similarly, in Table 1. H- and L-Ferritin Binding to Peripheral Blood Lymphocytes

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>cpm*</th>
<th>Positive Cells (%)</th>
<th>cpm*</th>
<th>Positive Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>750 ± 310</td>
<td>ND</td>
<td>840 ± 380</td>
<td>ND</td>
</tr>
<tr>
<td>Unstimulated (72-h culture)</td>
<td>2,770 ± 420</td>
<td>4.0 ± 1.2</td>
<td>860 ± 270</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>PHA-stimulated (72-h culture)</td>
<td>9,000 ± 880</td>
<td>26.8 ± 4.2</td>
<td>1,050 ± 390</td>
<td>1.9 ± 0.6</td>
</tr>
</tbody>
</table>

Values are the mean ± SD of three different experiments.

*1H-rHF and $^{35}$I-rLF binding.

†FACS results subtracted from background: rHF + MoAb 2A4 + GAM-Ig FITC; rLF + MoAb LF03 + GAM-Ig FITC.
H-Ft-BS increased steadily, but to a lesser degree, in the first 60 hours; then at 82 hours, it decreased to values similar to those of unstimulated cells.

Analyses of DNA cellular content in comparison with H-Ft-BS expression are shown in Fig 3. Computer processing of the DNA histogram shown in Fig 3A indicated that 59% of the cells were in S-phase and 3% were in G2+M-phase. The bivariate cytograms without and with fluores-
H-FERRITIN BINDING SITES ON HUMAN LYMPHOCYTES

Table 2. Dual-Color Flow Cytometry Analysis of H-Ft-BS and Other Surface Markers on PHA-Stimulated Lymphocytes

<table>
<thead>
<tr>
<th>Markers</th>
<th>H-Ft-BS of Marker +</th>
<th>H-Ft-BS of Marker +</th>
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<tbody>
<tr>
<td></td>
<td>of Marker +</td>
<td>Marker +</td>
</tr>
<tr>
<td>H-Ft-BS</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>CD4</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>CD8</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>CD19</td>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>IL-2-R</td>
<td>58</td>
<td>54</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>88</td>
<td>41</td>
</tr>
<tr>
<td>MLR3</td>
<td>71</td>
<td>41</td>
</tr>
<tr>
<td>T-8</td>
<td>74</td>
<td>42</td>
</tr>
</tbody>
</table>

Cells were analyzed after 60-hour culture in PHA.
*Percentage of total cell population expressing the surface marker.
†Fraction of the marker + cells coexpressing H-Ft-BS.
‡Fraction of H-Ft-BS+ cells coexpressing the marker.
§Value probably underestimated because of the large number of cells with very low antigen intensity.

DISCUSSION

We and other investigators previously demonstrated the existence of binding sites specific for H-ferritin (or H-rich ferritin) on various human cell lines.⁵⁶-⁵⁸ We showed that these binding sites also exist on normal human lymphocytes, where they have the same affinity constant and can reach a density of 20,000 sites per cell, similar to that of proliferating K562 cells.⁶ In addition, in lymphocytes, as in K562 cells, H-Ft-BS expression was modulated by cellular proliferation and was higher in the PHA-stimulated cells (Table 1).

We were unable to detect specific L-ferritin binding even in cells showing high binding of H-ferritin, which suggests that L-ferritin binding sites either do not exist on lymphocytes or are so different from H-Ft-BS that they are undetectable with the present method. The lack of rHF binding by fresh lymphocytes poses another problem because the binding sites may be either absent or masked. We favor the latter hypothesis because H-Ft-BS appeared after incubation in FCS medium, a treatment previously shown to shed ferritin from the lymphocyte surface,⁵⁸ and ferritin is present in the circulation where it may interact with the lymphocyte binding sites. Indeed, the proportion of circulating lymphocytes with membrane-bound ferritin (2% to 5%) detected in various laboratories³¹-³⁴ agrees well with the proportion that expressed H-Ft-BS after incubation in FCS (3% to 5%) in this study. Thus, even though an effect of incubation on H-Ft-BS expression cannot be ruled out, in the circulating cells H-Ft-BS probably is occupied by natural ferritin.

In PHA-stimulated lymphocytes, 30% of CD4 T-helper cells, 30% of CD8 T-cytotoxic suppressors, and most CD19 B lymphocytes expressed H-Ft-BS (Table 2) (the number of B cells analyzed was too small to establish that H-Ft-BS is a marker for all B lymphocytes). Thus, the distribution of the binding sites among lymphocytes is not related to cellular lineages, a result in keeping with previous data showing that H-Ft-BS exist on cells of varying origin, including liver, lung, and bone marrow.³⁵-³⁷ Although not related to cellular functional activities, H-Ft-BS expression appears to be closely and positively linked to the proliferative status of the lymphocytes for the following reasons: (a) total rHF binding, as well as the number of cells binding rHF, markedly increased after PHA-induced blastogenesis; (b) almost all cells expressing H-Ft-BS also expressed the activation markers IL-2-R, HLA-DR, MLR3, and T-8; (c) during PHA induction, the kinetics of H-Ft-BS expression was roughly parallel to that of the four activation markers; (d) H-Ft-BS exist on cells in various cell cycle phases, including actively proliferating cells in early S-phase.³⁵ These data are in accord with previous findings showing that H-Ft-BS do not exist in quiescent confluent K562 cells and increase markedly during cell proliferation.³⁶ In addition, H-Ft-BS expression shows a marked similarity to the behavior of CD30, a cluster of antigens of unknown function, accepted as a proliferation marker, which is expressed on K562 and HL60 cells and on about 20% of PHA-stimulated lymphocytes.³⁷ Its molecular weight is similar to that previously detected for H-Ft-BS.³⁸ Thus, we suggest that H-Ft-BS could be considered a proliferation marker.

The present study with ferritin H- and L-homopolymers confirms previous findings obtained with natural ferritins.
with different proportions of H:L [16-21]: ferritin has an inhibitory activity on lymphocyte blastogenesis that is associated with the H-chain, whereas the L-chain is noneffective. This ferritin activity on lymphocytes has analogies with the H-chain, whereas the L-chain is noneffective. This ferritin activity on lymphocytes has analogies with the activity exerted on myeloid precursors; in both cases, the suppression is partial and is linked to the presence of the H-chain, and both cell types express H-Ft-BS.

A recent study of BFU-E and CFU-GM showed that ferritin suppression results from ferroxidase activity of the H-chain because it disappears in the mutants with this activity deleted, and suppression is reversed by iron supplementation to cells in the form of hemin. Ferroxidase activity has been suggested to interfere with cellular proliferation by inducing iron starvation. A linkage between H-Ft-BS expression and iron metabolism is further suggested by the proportional expression of transferrin and ferritin receptors.

H-Ft-BS appears to behave unusually. It appears to be a proliferation marker with the function of downregulating proliferation. Whether it acts in concert with the Tf-R, which is also an activation marker, but with the function of supplying iron to cells, remains to be established.

REFERENCES

Fig 3. (A) DNA cell cycle distribution of PHA blasts at 72 hours. (B and C) Bivariate DNA (horizontal axis) with hHF-FITC (vertical axis); control sample (B) and stained sample (C). Green fluorescence (ie, presence of H-Ft-BS) was detectable throughout all cell cycle phases.

Fig 4. Inhibitory activity of H-ferritin. H- and L-ferritins were added in various concentrations to the culture medium with or without PHA. After 72 hours, 3H-thymidine incorporation was analyzed and results were expressed as PHA index*: (blastogenesis induced by PHA + ferritin - blastogenesis induced by ferritin)/(blastogenesis induced by PHA). Each point represents the mean of triplicate experiments with SE less than 10%. Experiments in which H-ferritin was incubated with 50 molar excess of anti-H-ferritin MoAb 2A4 before addition to the cells (△).


34. Fraker PJ, Speck JC: Protein end membrane iodination with a sparingly soluble chloroamide 1,3,4,6-tetrachloro 3a-6a diphenyl glycoluril. Biochem Biophys Res Commun 80:849, 1978


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