H- and L-Rich Ferritins Suppress Antibody Production, But Not Proliferation, of Human B Lymphocytes In Vitro

By Keiko Morikawa, Fumimaro Oseko, and Shigeru Morikawa

The effect of human spleen (L-rich) and heart (H-rich) ferritins on the proliferation and differentiation of human B lymphocytes was studied in comparison with that of holo- and apo-transferrins. Ferritins rich in H and L chain, as well as the transferrins, did not inhibit the proliferative response of resting and activated B cells stimulated with polyclonal B-cell mitogen, Staphylococcus aureus Cowan strain 1. In contrast, the ferritins, but not the transferrins, clearly suppressed the antibody production by B blasts in T-cell-independent as well as T-cell-dependent system. Kinetic study showed that inhibitory action of ferritins on immunoglobulin (Ig) production was caused at an early stage of B-cell differentiation. The cytoplasmic Ig-containing cells decreased in proportion to the reduction of Ig secretion. The evidence that ferritin inhibited Ig synthesis of Epstein-Barr virus-transformed human B-lymphoblastoid cell line also supported the idea that the effect of ferritin was directed toward the antibody-producing B lymphocytes. The molecular analysis showed that the inhibitory effect of ferritin was regulated at the transcriptional level of the Ig generation signal. Our results suggest that H- and L-rich ferritins exert their inhibitory action on the differentiation of B cells maturing into Ig-producing cells.

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MATERIALS AND METHODS

Cell preparation. Human tonsils were obtained by tonsillectomy from juvenile patients with chronic tonsillitis and were dispersed in single cell suspensions. Mononuclear cells (MNC) were isolated by Ficoll-Hypaque density gradients. Monocytes and natural killer cells were depleted by incubation with 5 mmol/L L-leucine methyl ester (Sigma, St. Louis, MO) in a serum-free medium. T cells were removed by rosetting twice with 2-aminoethylisothiouronium bromide (Sigma)-treated sheep erythrocytes (E). Nonrosetted (E-) cells were purified by isolating the cells from the interface at 40%/50%, 50%/60% and 60%/70% of a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden). The high-density fraction of B cells from the 60%/70% interface, designated as “resting B cells,” were used as target cells unless otherwise indicated.

Reagents. Human spleen and heart ferritins were purchased from UCB Bioproducts (Brussels, Belgium). Human spleen ferritin has a low content in H subunits (<15%) and its isoelectric point is in general basic (5.2 to 5.7). Human heart ferritin has a very high content of H subunit (50% to 60%) and its pl is in acidic (4.5 to 4.8). The purity of these ferritins is established by polyacrylamide gel electrophoresis, by analysis of their amino acid composition, and to analyze a series of B-cell proliferation and differentiation pathways. Using this system, the present study was undertaken to examine whether H- and L-rich ferritins have a distinct influence on the proliferation and differentiation of human B lymphocytes. Our results showed that H- and L-rich ferritins selectively inhibited the differentiation of B lymphocytes maturing into Ig-producing cells. In contrast, both ferritins did not suppress the proliferative response of B cells, regardless of their activation state.

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by a lack of detectable contamination by immunoelectrophoresis against an antiserum directed against whole human serum. Another ferritin preparation (human spleen), holo-iron-saturated- and apo-iron-unsaturated-transferrins, were obtained from Sigma. The reagents were dissolved in phosphate-buffered saline and further diluted in culture medium and added to the cultures. SAC and pokeweed mitogens (PWM) were obtained from Calbiochem Behring Diagnostics (La Jolla, CA) and GBICO Laboratories (Grand Island, NY), respectively. Recombinant human interleukin-2 (rhIL-2) was prepared in vitro activated B cells from high-density B cells by stimulating them with SAC and activated B lymphocytes. The ferritins did not interfere with the proliferative response of these in vivo activated B cells (Table 1). We further examined the effects of ferritin on the low proliferative response of human B lymphocytes. High-density B cells from tonsillar samples, which were designated as "resting" B cells, were cultured for 3 days with SAC (1:10^6 vol/vol). Both ferritins did not interfere with the proliferative response of resting B lymphocytes at concentrations of 0.8 to 100 ng/mL (Table 1). Next, we prepared in vitro activated B cells from high-density B cells by stimulating them with SAC for 3 days, and we recultured them in the presence or absence of the ferritins for an additional 3 days with or without rhIL-2 (100 U/mL). Both ferritins did not interfere with the proliferative response of in vitro activated B cells (Table 1). We further examined the effect of ferritin on the low (40%/50% interface) and intermediate (50%/60% interface) densities of B cells, which were regarded as in vivo-activated B cells. The ferritins did not inhibit the proliferation of these in vivo activated B cells (data not shown). These data indicate that both H- and L-

**RESULTS**

**Effect of ferritins on the proliferative response of resting and activated B lymphocytes.** Human spleen (L-rich, basic) and heart (H-rich, acidic) ferritins were examined for their effects on the proliferative response of human B lymphocytes. High-density B cells from tonsillar samples, which were designated as "resting" B cells, were cultured for 3 days with SAC (1:10^6 vol/vol). Both ferritins did not interfere with the proliferative response of resting B lymphocytes at concentrations of 0.8 to 100 ng/mL (Table 1). Next, we prepared in vitro activated B cells from high-density B cells by stimulating them with SAC for 3 days, and we recultured them in the presence or absence of the ferritins for an additional 3 days with or without rhIL-2 (100 U/mL). Both ferritins did not interfere with the proliferative response of in vitro activated B cells (Table 1). We further examined the effect of ferritin on the low (40%/50% interface) and intermediate (50%/60% interface) densities of B cells, which were regarded as in vivo-activated B cells. The ferritins did not inhibit the proliferation of these in vivo activated B cells (data not shown). These data indicate that both H- and L-

**Table 1. Effect of Basic and Acidic Ferritins on the Proliferative Response of Human B Cells**

<table>
<thead>
<tr>
<th>Ferritin</th>
<th>Concentration (ng/mL)</th>
<th>Resting B</th>
<th>Activated B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic</td>
<td>0</td>
<td>0.3 ± 0.1</td>
<td>18.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.3 ± 0.2</td>
<td>18.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.3 ± 0.1</td>
<td>21.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.0 ± 0.0</td>
<td>20.0 ± 0.6</td>
</tr>
<tr>
<td>Acidic</td>
<td>0.8</td>
<td>1.1 ± 0.1</td>
<td>17.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.3 ± 0.1</td>
<td>20.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.9 ± 0.1</td>
<td>19.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.1 ± 0.1</td>
<td>16.8 ± 0.5</td>
</tr>
</tbody>
</table>

**Table 2. Effect of Ferritins and Transferrins on Human B-Cell Proliferation**

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>L-Ferrin®</th>
<th>L-Ferrin®</th>
<th>Holo-Transferrin</th>
<th>Apo-Transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>29.0 ± 0.6</td>
<td>30.6 ± 0.6</td>
<td>28.3 ± 0.7</td>
<td>28.5 ± 0.8</td>
</tr>
<tr>
<td>0.8</td>
<td>30.0 ± 0.7</td>
<td>31.3 ± 0.2</td>
<td>28.7 ± 0.1</td>
<td>29.0 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>31.4 ± 0.3</td>
<td>32.6 ± 0.9</td>
<td>28.6 ± 0.4</td>
<td>29.9 ± 0.4</td>
</tr>
<tr>
<td>20</td>
<td>33.2 ± 0.5</td>
<td>30.4 ± 1.0</td>
<td>29.1 ± 0.2</td>
<td>29.8 ± 0.6</td>
</tr>
<tr>
<td>500</td>
<td>32.0 ± 0.3</td>
<td>31.0 ± 0.8</td>
<td>29.9 ± 0.2</td>
<td>31.2 ± 0.9</td>
</tr>
</tbody>
</table>

**Nordern blot analysis.** The total cellular RNA was isolated by the guanidine thiocyanate procedure and 20 μg of the RNA was electrophoresed through 1.2% agarose gel and transferred to nylon membranes. Northern blot analysis with randomly-primed [32P]-labeled DNA probe was performed as described.25 Plasmid containing the appropriate human μDNA probe was isolated by alkaline lysis and subjected to Sepharose CL-6B chromatography. Appropriate DNA insert was isolated from the plasmid by restriction endonuclease digestion and fragment from plasmid pGEM containing human germine heavy-chain constant-region C1-C3. Autoradiographic signals were quantitated by densitometric scanning of Fuji photographs (Super HR-L; Fuji Photo Film Co, Ltd, Kanagawa, Japan) of Northern blots taken under a LKB ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden).

**Cell line.** Ysm 3B1, an Epstein-Barr virus (EBV)-transformed human B-lymphoblastoid cell line, has been established in our laboratory and has been maintained in RPMI 1640 supplemented with 10% FCS. The Ysm 3B1 cells spontaneously secrete IgM and IgG in the culture supernatants.
rich ferritins do not affect the proliferative response of human B lymphocytes, regardless of their activation state.

**Comparative study of the effect of ferritins and transferrins on human B-cell proliferation.** To make sure of the effect of ferritins on the B-cell proliferation, the effect of a different ferritin preparation (Sigma), holo- and apo-transferrins was compared with that of L-rich ferritin (UCB Bioproduct). As shown in Table 2, any of the ferritin and transferrin inhibited the B-cell proliferation induced by SAC during the 3-day period at concentrations of 0.8 to 500 ng/mL. These results indicate that both ferritins and transferrins do not disturb the proliferative response of human B lymphocytes.

**Effect of the ferritins on Ig generation by B cells.** We examined the effect of ferritins on the Ig synthesis by human B lymphocytes. Tonsillar MNC were cultured with PWM (1:25 vol/vol) in the presence of the ferritins for 7 days and the amounts of Ig secreted in the culture supernatants were measured by ELISA. Both ferritins suppressed Ig generation of B cells in a dose-dependent manner at concentrations of 0.16 to 500 ng/mL, though the inhibitory activity of H-rich
(heart) ferritin was slightly greater than that of L-rich (spleen) ferritin. The result of IgG suppression by these ferritins in PWM-driven system was shown in Fig 1A. As PWM-driven Ig generation system depends on T cells and macrophages or monocytes, we further examined the effect of these ferritins on the Ig generation in T cell-independent system. The B cells were stimulated with SAC for 48 hours, then washed thoroughly and recultured in the presence or absence of rhIL-2 for another 7 days. The presence of rhIL-2 induced significant Ig production by B cells in the SAC-induced system. Addition of the ferritins into the cultures at concentrations of 0.16 to 500 ng/mL resulted in a reduction of Ig secretion in a very similar fashion as observed in PWM-driven system as shown in Fig 1 (B). These results suggest that both H- and L-type ferritins directly suppressed the antibody-generating capacity of B lymphocytes.

**Effect of ferritins and transferrins on the differentiation of B cells.** To examine whether the inhibitory action on the antibody generation is specific for the ferritin, the effect of L-rich ferritin was compared with those of a different ferritin preparation, holo- and apo-transferrins in T-cell-independent system. As shown in Fig 2, the two ferritins, but not transferrins, clearly suppressed IgM and IgG generation by B blasts in a dose-dependent manner. Thus, it is unlikely that the inhibitory effect of ferritins on the B-cell differentiation was induced by nonspecific event.

**Time-course analysis of the effect of ferritin on Ig secretion.** Figure 3 shows the inhibitory action of L-ferritin when added at different time intervals over 7 days at the concentrations causing marked reduction of Ig secretion (100 ng/mL) in SAC-induced Ig generation system. The presence of the ferritin throughout the 7-day culture showed its maximal inhibitory action in IgG and IgM generations by activated B cells, and addition of the ferritin at 2 days after culture initiation had a similar effect. However, ferritin was relatively ineffective when added to the culture at day 5 or later. These data imply that ferritin was involved in an early phase of B-cell differentiation to antibody-producing cells that completes within 2 days.

A **dose-dependent decrease in the number of cytoplasmic Ig-positive (clg<sup>+</sup>) B cells by ferritin.** The time-course study indicated that ferritin suppressed Ig production of B cells at an early phase of B-cell differentiation to antibody-producing cells. In this experiment, it was further examined whether the ferritin affects the Ig synthesis at a cytoplasmic level in B cells. Addition of ferritin in the SAC-induced B-cell differentiation system markedly decreased the frequency of clg<sup>+</sup> cells at concentrations of 0.8 to 100 ng/mL in a dose-dependent manner (Table 3). The decrease in the percentage of clg<sup>+</sup> cells was roughly proportional to the inhibitory levels of Ig secretion in culture supernatants. These results indicate that ferritin exerts its inhibitory activity on Ig generation rather than Ig secretion.

**Inhibitory activity on Ig generation of EBV-transformed human B-lymphoblastoid cell line shown by ferritins.** The above data showed that both H- and L-rich ferritins inhibit Ig generation, but not proliferation, of normal human B lymphocytes. Then, it was examined whether the ferritin has a similar inhibitory effect to the proliferation and Ig production by an EBV-transformed human B-lymphoblastoid cell line. Yam 3B1 is an EBV-transformed B-cell line that spontaneously secretes IgM and IgG in the culture supernatants. When these cells were cultured in the presence of ferritin, ferritin clearly suppressed Ig production as well as proliferation at concentrations of 0.8 to 100 ng/mL in a

<table>
<thead>
<tr>
<th>Addition of L-Ferritin (ng/mL)</th>
<th>clg&lt;sup&gt;+&lt;/sup&gt; Cells (%)</th>
<th>IgG Secretion (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17.5</td>
<td>911</td>
</tr>
<tr>
<td>0.8</td>
<td>15.1</td>
<td>816</td>
</tr>
<tr>
<td>4</td>
<td>10.5</td>
<td>822</td>
</tr>
<tr>
<td>20</td>
<td>7.9</td>
<td>288</td>
</tr>
<tr>
<td>100</td>
<td>3.7</td>
<td>101</td>
</tr>
</tbody>
</table>

SAC-activated B cells (2 x 10<sup>6</sup>/well) were cultured with L-ferritin in the presence of rhIL-2 (100 U/mL) for 7 days. Then, the slides were prepared using a cytocentrifuge, fixed and were stained with FITC-conjugated antihuman Ig, clg<sup>+</sup> cells were detected by fluorescence microscopy. The percentage was determined by counting at least 400 cells. Amounts of Ig secreted in the culture supernatants were measured by ELISA. The values represent the mean of triplicate cultures. The values of control cultures are 3.1% of clg<sup>+</sup> cells, and 113 ng/mL and 169 ng/mL of IgM and IgG secretion, respectively.
dose-dependent fashion (Table 4). Though the process of normal B-cell maturation is divided into the proliferation and differentiation stages in the in vitro experiment, it is impossible to separate the activation process of the cultured cell line into these two stages, because they concomitantly proliferate and differentiate. Namely, the data indicate that the action of the ferritin was directed toward the inhibition of B-cell differentiation, not induced as a consequence of the prolongation of the proliferative response.

**Ferritin suppressed the expression of mRNA in antibody-producing B cells.** Because treatment of B cells with ferritin caused the decrease in Ig generation, we tested the effect of ferritin on the mRNA expression in antibody-producing B cells by using the CSDNA probe as described previously. Total RNA was collected at day 4 from the SAC-prestimulated B cells cultured either in medium, in spleen ferritin (100 ng/mL), IL-2 (100 U/mL) or in IL-2 plus ferritin. Subsequently, 20 µg of RNA were processed for Northern blot analysis and hybridized with the CSDNA probe. Untreated B cells showed small amounts of mRNA expression. B cells cultured with ferritin alone showed the reduction of mRNA expression compared with that of untreated cells. The mRNA accumulation in B cells cultured with IL-2 (100 U/mL) was pronounced, and the mRNA accumulation was dramatically decreased in B cells cultured with IL-2 and ferritin as shown in Fig 4. The relative peak densities of autoradiograms showed that the ratio of mRNA signal in untreated and ferritin-treated B cells were low (0.082 and 0.028, respectively), and B cells treated with IL-2 was relatively high (0.302), and it was greatly decreased in the presence of ferritin to the level of the untreated B cells (0.035). These results indicate that the inhibitory effect of ferritin on Ig generation is regulated at the transcriptional level of Ig generation signal.

**DISCUSSION**

The present investigation showed that both H- and L-rich ferritins suppressed Ig generation rather than proliferation of human B lymphocytes. Ferritins exerted their inhibitory activity on Ig generation by B lymphocytes in T-cell–independent (SAC-activated) as well as T-cell–dependent system (a PWM-driven system). Although Ig production by B cells depends on T cells and macrophage/momocytes in the PWM-driven system, B cells require T-cell–derived factor to produce Ig in a SAC-activated system. As both holo- and apo-transferrins do not have such activity, and a different ferritin preparation also showed the activity, the inhibitory activity of ferritin on Ig synthesis is unlikely to be nonspecific event. A kinetic study suggests that inhibitory action of the ferritin in Ig generation was caused at an early stage of B-cell differentiation into antibody-producing cells. In the presence of ferritin, cytoplasmic Ig-containing cells decreased in proportion of the reduction of Ig secretion (Table 3). From these results, we consider that the effect of ferritin is directed toward the B lymphocyte maturing into Ig-producing cells. In spite of the suppressive effect on antibody production, both ferritins could not suppress the proliferative response of B cells, regardless of the B-cell activation state. In addition, the ferritins did not have cytotoxic activity when the cell viability was examined by trypan blue dye exclusion method (data not shown). Ig generation of an EBV-transformed human B-lymphoblastoid cell line, which spontaneously secretes Ig in their culture medium, was suppressed in the presence of ferritin in the culture medium (Table 4). The finding that ferritin inhibited the proliferation of this cell line suggests that the prevention of the terminal differentiation by ferritin is not induced as a consequence of the prolongation of the cell proliferation. When the effects of ferritin on the mRNA expression in antibody-producing B cells were examined by Northern blot analysis,
the ratio of \( \mu \text{mRNA} \) signal intensity in B cells treated with IL-2 was markedly high, but that of the B cells treated with ferritin and IL-2 decreased to the level of control B cells when estimated densitometrically (Fig 4). These results suggest that the effect of ferritin on Ig generation by B cells was regulated at the transcriptional level. As ferritin suppresses T-cell proliferation induced by polyclonal T-cell mitogen,\(^{16,17} \) the effect of ferritin on B-cell activation seems to be distinct from that on T-cell function.

The inhibitory action of ferritin is associated with the H chain, whereas the L chain does not act to inhibit the colony formation by hematopoietic progenitor cells\(^{8-13} \) and the PHA-induced blastogenesis of T cells.\(^{23} \) However, the immunosuppressive effect on T-lymphocyte function has also been induced by L-rich ferritins from liver,\(^{14} \) spleen\(^{15,16,18} \) and placenta.\(^{17} \) In addition, a role for acidic, but not basic, ferritin as inhibitor of myelogenesis is not convincing, too.\(^{8,10,13} \) There was contradictory information in order to establish a specific role for acidic ferritin in the control of hematopoiesis and lymphocyte function. In our study, the suppressive activity of L-rich ferritin on Ig production was not different with that of H-rich ferritin, implying that the effect of ferritin on the differentiation of B cells may not be linked with either of the ferritin type in our study. However, natural ferritins used in this study have different proportions of H:L ratio. Therefore, it is less clear from this study whether the inhibitory effect of ferritin on lymphocyte function is linked to the presence of the H chain, until the experiment using recombinant ferritin preparations is undertaken.

Both H- and L-type ferritin binding sites are found in T- and B-lymphoid cell lines.\(^{21} \) Some investigators describe that specific binding sites for L ferritin are unable to detect, although H ferritin specific binding sites exist, in normal human lymphocytes.\(^{23} \) Interestingly, H-ferritin binding sites are present on the cells expressing the activation markers such as HLA-DR, CD25 and CD71, though the binding sites are not connected with cell lineage or functions.\(^{21} \) Thus, H-ferritin binding sites appear to be closely linked to the activation states of the lymphocytes. In our study, we showed that ferritins did not interfere with the proliferative response of the activated B cells in vivo as well as in vitro. Whereas, other studies indicate that a ferritin rich in L subunits also binds to T- and B-lymphoid cells independently of the binding of H subunits.\(^{21} \) These lines of evidence suggest that a linkage between the expression of H-ferritin binding sites and immunosuppressive activity of ferritin is not fully conclusive.

Small amounts of ferritin are found in the sera of normal individuals, although serum ferritin is predominantly basic.\(^{26} \) In the present study, very low concentrations of ferritin, which were at physiologic values,\(^{26} \) caused significant inhibition of Ig secretion in the in vitro assay system. Though the possible physiologic significance of the inhibitory activity of ferritin on the B-cell activation remains to be uncertain, it is probable that this activity has been blocked or overwhelmed by the various factors or cells in vivo. However, clinical observations associated with hyperferritinemia, especially in relation to the immunologic status in patients with malignancies\(^{26-28} \) suggested that ferritin may participate in the development of impaired cell-mediated immunity. The present study indicates that not only T lymphocytes, but also B lymphocytes may play a significant role in this event.

REFERENCES

16. Kein P, Descamps-Latscha B: In vitro suppression of cell-


H- and L-rich ferritins suppress antibody production, but not proliferation, of human B lymphocytes in vitro

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