Isolated rat hepatocytes acquire iron from lactoferrin by endocytosis

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The iron-binding protein lactoferrin (Lf) present in blood is metabolized by the liver. Isolated rat hepatocytes vigorously endocytose bovine Lf via recycling Ca^{2+}-dependent binding sites, but the uptake of iron from Lf by hepatocytes has not been examined. In this study, isolated rat hepatocytes were incubated with radiolabelled bovine Lf (^{125}I-Lf, ^{59}Fe-Lf or ^{125}I-^{59}Fe-Lf) at 37 °C, then washed at 4 °C in the presence of dextran sulphate with either Ca^{2+} or EGTA to distinguish between total bound and internal radioactivity respectively. Cells internalized ^{125}I-Lf protein and Lf-bound ^{59}Fe at maximal endocytic rates of 1700 and 480 mol·cell^{-1}·s^{-1} respectively. When Lf was normalized for ^{59}Fe content, these endocytic rates were equivalent and reflected an uptake potential of at least 3400 mol of iron·cell^{-1}·s^{-1}. Cells prebound with ^{125}I-^{59}Fe-Lf to Ca^{2+}-dependent sites at 4 °C internalized more than 80% of both ^{125}I-Lf protein and Lf-bound ^{59}Fe approx. 6 min after warming to 37 °C at similar rates (^{125}I-Lf: $k_{in} = 0.276$ min^{-1}, ^{59}Fe: $k_{in} = 0.303$ min^{-1}). Within 4 h at 37 °C, cells had released 25% or less internalized Lf protein in the form of acid-soluble ^{125}I-by-products but retained all the Lf-delivered ^{59}Fe. Hyperosmotic disruption of clathrin-dependent endocytosis blocked the uptake of ^{125}I-Lf and Lf-bound ^{59}Fe. Incubation of cells with ^{125}I-^{59}Fe-Lf and a 100 molar excess of diferric transferrin reduced slightly the endocytosis of ^{125}I-Lf protein and ^{59}Fe accumulation. Treatment of cells with the ferric chelator desferrioxamine did not alter uptake of ^{125}I-Lf protein or Lf-bound ^{59}Fe, but the ferrous chelator bathophenanthroline disulphonate slightly elevated endocytosis of ^{125}I-Lf protein and Lf-bound ^{59}Fe. These findings indicate that Lf does not release its bound iron before endocytosis. It was concluded from this study that hepatocytes take up iron from Lf at high rates by a process that requires endocytosis of Lf–iron complexes.

INTRODUCTION

As the primary iron-processing organ in mammalian systems, the liver receives much of the iron retrieved during the turnover of senescent erythrocytes by the reticuloendothelial system. Various blood proteins, including transferrin (Tf), haemopexin, haptoglobin and serum ferritin, ferry non-haem and haem-associated iron to the liver where they are endocytosed, primarily by hepatocytes [1,2]. Hepatocytes may also actively import iron–salt chelates directly into the cytoplasm at the plasma membrane [3]. By sorting mechanisms that are poorly understood, hepatocytes route this iron to intracellular ferritin stores, mitochondria or endoplasmic reticulum (haem) or package it with newly synthesized Tf for export out of the liver (for a review, see [1]).

The liver also clears exogenous lactoferrin (Lf) and its bound iron injected into the blood [4], a process performed primarily by hepatocytes [5–7]. The role of Lf in hepatic iron metabolism, however, is unclear. Isolated rat hepatocytes bind bovine Lf at 4 °C by sets of high- and low-affinity binding sites, the former of which are Ca^{2+}-dependent [8]. Lf bound to the Ca^{2+}-dependent high-affinity sites can be selectively removed by washing cells with EGTA. Lf bound to the low-affinity sites can be selectively removed by washing cells in the polyanion dextran sulphate. Complete removal of more than 95% of surface-bound Lf requires washing cells with both EGTA and dextran sulphate. At 37 °C, hepatocytes endocytose Lf only by the Ca^{2+}-dependent binding sites [9]. Hepatocytes possess ~ 1 million Ca^{2+}-dependent Lf-binding sites, most of which are at the cell surface under steady-state conditions. Via these sites, hepatocytes bind, internalize and degrade Lf regardless of the protein's iron content. Hepatocytes recycle the Lf-binding sites within 5 min, using them in additional rounds of Lf endocytosis.

The high number and endocytic capacity of these recycling Lf-binding sites on hepatocytes lend support to the notion that Lf in blood scavenges iron to the liver. To date, however, Lf-dependent iron uptake by isolated hepatocytes has not been examined. Thus the purpose of this study was twofold: first, to characterize the extents and rates of iron uptake by isolated rat hepatocytes from bovine Lf in order to understand better the capacity of hepatocytes to incorporate iron from Lf; secondly to examine the effect of endocytosis inhibitors and iron chelators on Lf-mediated iron delivery to determine the specific uptake pathway hepatocytes use to incorporate iron from Lf. The findings provide evidence that isolated rat hepatocytes incorporate iron from Lf vigorously, a process that requires the endocytosis of Lf.

EXPERIMENTAL

Materials

Bovine colostrum Lf, BSA (fraction V), desferrioxamine (DFO), dextran sulphate (5000 Da), Hepes and rat diferric Tf were purchased from Sigma (St. Louis, MO, U.S.A.). Collagenase (types A and D) was obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Na^{125}I (~ 17 Ci/mg of iodine) was from ICN Biomedicals (Irvine, CA, U.S.A.), Amersham (Arlington Heights, IL, U.S.A.) or DuPont–NEN (Boston, MA, U.S.A.), $^{59}FeCl_3$ (1 mCi/ ~ 130 μg Fe) was from ICN and ^{59}Fe(II) citrate was from DuPont–NEN. All other chemicals were reagent grade. Basal medium Eagle's (BME) was obtained

Abbreviations used: BPS, bathophenanthroline disulphonate; BME, basal medium Eagle's; DFO, deferoxamine mesylate (desferrioxamine); HBS, Hepes-buffered saline; ^{125}I-^{59}Fe-Lf, ^{125}I-[^{59}Fe]lactoferrin; Tf, transferrin.

from Sigma. Hepes-buffered saline (HBS) contained 150 mM NaCl, 3 mM KCl and 10 mM Hepes, pH 7.4. BME was supplemented with 2.4 g/l Hepes, pH 7.4, and 0.22 g/l $NaHCO_3$. BME/BSA is BME containing 0.1 % (w/v) BSA.

Hepatocytes

Male Sprague–Dawley rats (150–350 g; Harlan Labs, Indianapolis, IN, U.S.A.) were fed on standard laboratory chow and water *ad libitum*. Hepatocytes were prepared by a modification of a collagenase perfusion procedure [10] as described previously [8,11]. Cells were kept at ~ 30 °C during the filtration and differential centrifugation steps. Final cell pellets suspended in ice-cold BME/BSA were ~ 85 % viable and single cells. Before experiments, cell suspensions (2×10^6–4×10^6 cells/ml in BME/BSA; 10 % of the flask volume) were incubated at 37 °C for 60 min to allow recovery from the isolation procedure. Viability was determined microscopically by Trypan Blue exclusion.

Lf preparation

Bovine colostrum LF (commercial preparation more than 90 % pure electrophoretically) at 5 mg/ml in 100 mM KCl/10 mM KH_2PO_4, pH 7.2, was further purified by ion-exchange chromatography as described previously [8]. Apo-Lf was prepared by dialysis of Lf against citric acid [12]. Holo-Lf was prepared by dialysis of Lf against Na_2CO_3/sodium citrate followed by incubation with $FeCl_3$ [13]. Purified apo- and holo-Lf were dialysed against HBS, filter-sterilized (0.2 μm) and stored at −20 °C before use. The iron contents, determined spectroscopically [14,15] from the A_{465}/A_{280} ratio, were routinely ~ 0.002 and ~ 0.04 respectively for apo-Lf and holo-Lf preparations. ^{125}I-Lf, prepared by the Iodogen method [16], had specific radioactivities of 3–65 d.p.m./fmol. ^{59}Fe-Lf was prepared by incubating apo-Lf (2.5 mg) in 0.5 ml of 0.1 M sodium citrate/0.1 M Na_2CO_3, pH 8.6, with $^{59}FeCl_3$ or ^{59}Fe(II) citrate ($\leqslant$ 200 μCi). ^{59}Fe-Lf was desalted over a GF-5 (Pierce) column and either dialysed against HBS or washed by ultrafiltration (10 kDa cut-off; Amicon) to remove free ^{59}Fe. In some instances, ^{59}Fe-Lf was iodinated as described above to yield double-labelled (^{125}I-^{59}Fe-) Lf. ^{59}Fe-Lf and ^{125}I-^{59}Fe-Lf preparations had specific radioactivities of 600–950 d.p.m./pmol and contained 0.14–1.4 mol of ^{59}Fe/mol of Lf. Homogeneity of radiolabelled and unlabelled Lfs used for all binding studies was confirmed by SDS/PAGE and autoradiography. ^{59}Fe-Lf preparations were used within 3 weeks of labelling. Apo- and holo-Lf prepared as described above bind hepatocytes specifically and with high affinity and compete with each other fully for binding to hepatocytes [8,9].

Lf binding and endocytosis assays

For Lf-binding assays, 2×10^6–4×10^6 cells/ml were routinely incubated with ^{125}I-Lf, ^{59}Fe-Lf or ^{125}I-^{59}Fe-Lf at 4 °C for 90 min in either BME/BSA or HBS supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. Cells were washed free of unbound radiolabelled ligand by centrifugation twice in excess HBS containing 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.1 % (w/v) dextran sulphate (HBS/Ca/DS) (10 min per wash). After being washed, cells were resuspended in 0.5–1.0 ml of HBS/Ca/DS, transferred to clean plastic tubes and assayed for radioactivity and protein. To measure hepatocyte endocytosis of ^{125}I-Lf, ^{59}Fe-Lf or ^{125}I-^{59}Fe-Lf, 2×10^6–4×10^6 cells/ml of BME/BSA were incubated with Lf at 37 °C as described in the Figure legends. Uptake was stopped rapidly by diluting the cells into a 5–10-fold excess volume of ice-cold HBS/Ca/DS. To assay total cell-associated (surface and intracellular) Lf, cells were washed by centrifugation twice in 2–4 ml of cold HBS/Ca/DS. To assay internalized Lf only, cells were washed by centrifugation twice (10 min each wash) in 2–4 ml of HBS containing 5 mM EGTA and 0.1 % (w/v) dextran sulphate (HBS/EDTA/DS) at 4 °C. After being washed, cells were resuspended in 0.5–1.0 ml of HBS/Ca/DS, transferred to clean plastic tubes and assayed for radioactivity and protein.

General

Protein was determined by the bicinchoninic acid protein assay procedure [17] using BSA as standard (Pierce Chemical Co.). Hepatocytes contained 1.1 mg of protein/10^6 cells. Centrifugation of cell suspensions was at 400 *g* for 2 min at 4 °C (Sorvall RT6000B centrifuge; DuPont Company, Wilmington, DE, U.S.A.). ^{125}I and ^{59}Fe radioactivities were determined using a Packard Çobra Auto-Gamma Counting System (model 5002; Packard Instrument Co., Downers Grove, IL, U.S.A.). Osmolality was determined using a Wescor 5500 vapour pressure osmometer (Wescor, Logan, UT, U.S.A.). Spectral analysis of Lf was performed using a Beckman DU-64 spectrophotometer. Statistical analysis included one-way analysis of variance or analysis of covariance followed by *post-hoc* Turkey's tests ($\alpha = 0.05$) using Systat 5.2 software (Macintosh).

RESULTS

Kinetics of Lf-bound iron uptake

Hepatocytes vigorously bind and internalize Lf protein by a clathrin-dependent endocytic pathway [8,9]. Thus I determined initially the extent to which hepatocytes accumulated ^{59}Fe from Lf as well as their uptake of ^{125}I-Lf protein. Cells were incubated in the continuous presence of an excess of ^{125}I-^{59}Fe-Lf at 4 and 37 °C and assayed subsequently for the amount of bound and internalized Lf protein (^{125}I) and Lf-bound iron (^{59}Fe) (Table 1). Under these conditions, hepatocytes bound Lf protein and Lf-bound iron similarly. At 4 °C, a temperature at which endocytosis is blocked, HBS/EGTA/DS removed 90 and 77 % of total bound ^{125}I and ^{59}Fe respectively from the cells. After incubation at 37 °C, cells bound ~ 9-fold more ^{125}I and ^{59}Fe than they did at 4 °C and retained ~ 85 % of their associated radioactivity after the HBS/EGTA/DS wash. Thus hepatocytes incorporated ^{59}Fe from Lf in a temperature-dependent manner in parallel with endocytosis of Lf protein. These data also confirmed that washing cells with HBS/EGTA/DS effectively removed surface-bound ^{125}I-Lf and Lf-bound ^{59}Fe.

Table 1 Binding and internalization of ^{125}I-^{59}Fe-Lf

Hepatocytes were incubated with ^{125}I-^{59}Fe-Lf (5 μg/ml) at 4 or 37 °C for 45 min, then assayed for bound and internalized radioactivity (1.8×10^6 cells/sample) as described in the Experimental section. Values represent means ± ranges of duplicate samples. Values in parentheses represent the percentage of total bound radioactivity that was resistant to HBS/EGTA/DS stripping (internal). The ^{125}I-^{59}Fe-Lf preparation used in this experiment contained 0.94 mol of ^{59}Fe/mol of Lf.

		^{125}I-^{59}Fe-Lf bound (pmol/sample)	
Temperature (°C)	Probe	Total	Internal
4	^{125}I	0.765 ± 0.005	0.075 ± 0.005 (9.8)
37	^{125}I	7.685 ± 0.005	7.025 ± 0.245 (91.4)
4	^{59}Fe	0.555 ± 0.125	0.13 ± 0.07 (23.4)
37	^{59}Fe	5.175 ± 0.085	4.395 ± 0.055 (84.9)

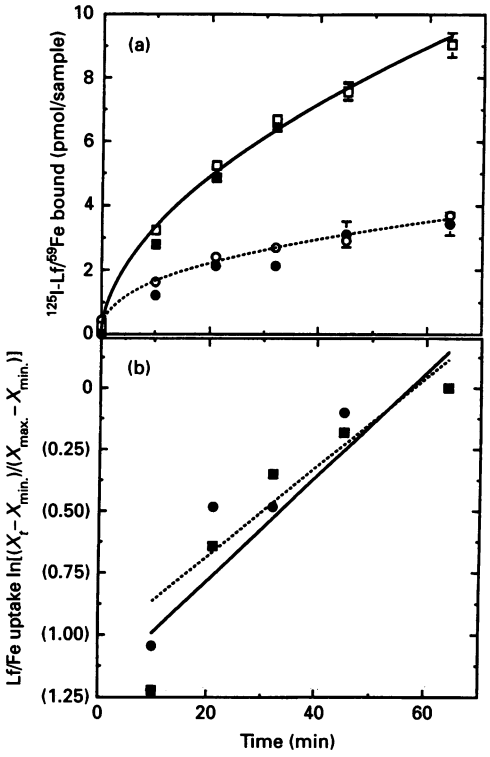

Figure 1 Kinetics of ^{125}I-^{59}Fe-Lf uptake

(**a**) Cells were incubated at 37 °C with ^{125}I-^{59}Fe-Lf (10 μg/ml; ^{59}Fe/Lf molar ratio = 0.28), and samples (1.9×10^6 cells) were assayed periodically for total bound (□, ○) and internal (■, ●) ^{125}I (□, ■) and ^{59}Fe (○, ●) radioactivity as described in the Experimental section. Symbols and error bars represent means ± ranges of duplicate samples. Best-fit isotherms were calculated according to the equations $y = 0.948x^{0.55}$ (^{125}I-Lf protein) and $y = 0.636x^{0.417}$ (^{59}Fe). (**b**) Uptake data were subjected to first-order rate analysis according to the equation

$$\ln \frac{X_t - X_{min.}}{X_{max.} - X_{min.}} = k_{in} t,$$

where X_t is radioactivity uptake at time t, $X_{min.}$ is radioactivity uptake at $t = 0$, $X_{max.}$ is maximal radioactivity uptake during the time course, and k_{in} is the first-order rate constant. ^{125}I-Lf: $k_{in} = 0.021$ min^{-1}, $r = 0.93$; ^{59}Fe: $k_{in} = 0.018$ min^{-1}, $r = 0.93$. ■, Lf; ●, Fe. Parenthetic values are negative numbers.

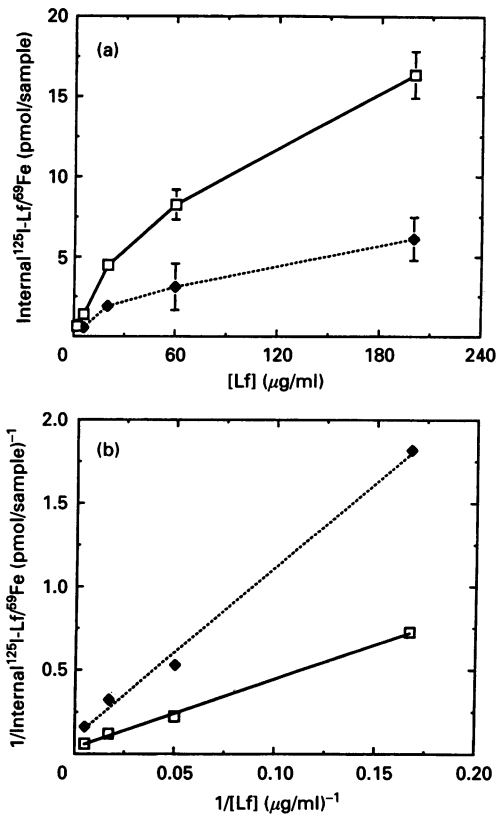

Figure 2 Determination of maximal endocytic rates for ^{125}I-^{59}Fe-Lf

(**a**) Cells were incubated with ^{125}I-^{59}Fe-Lf ($\leqslant$ 200 μg/ml; ^{59}Fe/Lf molar ratio = 0.28) at 37 °C for 1 h, then assayed for internalized radioactivity as described in the Experimental section. Symbols and error bars represent means ± ranges of duplicate samples. (**b**) Data from (**a**) were analysed by double-reciprocal plot to determine maximal endocytic rates: ^{125}I-Lf protein: 1700 mol·cell^{-1}·s^{-1}, $r = 0.993$; ^{59}Fe: 480 mol·cell^{-1}·s^{-1}, $r = 0.996$. □, ^{125}I-Lf; ◆, ^{59}Fe.

I also examined the kinetics of ^{59}Fe-Lf uptake by hepatocytes. When incubated at 37 °C in the continuous presence of excess ^{125}I-^{59}Fe-Lf, hepatocytes bound and rapidly internalized ^{125}I-Lf protein and Lf-bound ^{59}Fe at average rates of 713 and 270 mol·cell^{-1}·s^{-1} (Lf/^{59}Fe ratio = 0.28; Figure 1a). Regardless of the duration of the 37 °C incubation, ~ 74% of the cell-associated ^{59}Fe was resistant to the HBS/EGTA/DS wash and deemed to be intracellular. Hepatocytes bound and internalized ^{125}I-Lf in a similar manner, and ~ 72% of cell-associated ^{125}I-Lf was intracellular at all time points assayed. First-order rate analysis of these data (Figure 1b) showed that hepatocytes internalized ^{59}Fe and ^{125}I-Lf at statistically identical rates ($P = 0.635$; ^{125}I-Lf: $k_{in} = 0.021$ min^{-1}, $r = 0.93$; ^{59}Fe: $k_{in} = 0.018$ min^{-1}, $r = 0.93$).

I determined the maximal rates of hepatocyte uptake of ^{59}Fe from Lf by measuring ^{125}I-^{59}Fe-Lf uptake as a function of ligand concentration (Figure 2). Hepatocytes accumulated both ^{125}I-Lf protein and Lf-bound ^{59}Fe in a saturable manner with similar dose–responses (Figure 2a). Double-reciprocal analysis of these uptake data (Figure 2b) indicated that hepatocytes accumulated ^{125}I-Lf and ^{59}Fe at maximal rates of 1700 and 480 mol·cell^{-1}·s^{-1} respectively. Because ^{125}I-^{59}Fe-Lf contained 0.28 mol of ^{59}Fe/mol of ^{125}I-Lf, the maximal rate of ^{59}Fe uptake by hepatocytes was as expected if cells internalized ^{125}I-Lf protein and Lf-bound ^{59}Fe by the same mechanism ($480 \div 1700 = 0.282$). These data also indicated that, as Lf possesses two iron-binding sites per molecule, it could donate ~ 3400 iron molecules·hepatocyte^{-1}·s^{-1} if Lf was iron-saturated.

To determine the uptake rate constants for Lf-bound ^{59}Fe, I examined the kinetics of a single wave of ^{125}I-^{59}Fe-Lf internalization. To do this, I prebound cells with ^{125}I-^{59}Fe-Lf at 4 °C then removed unbound ^{125}I-^{59}Fe-Lf and ^{125}I-^{59}Fe-Lf bound to Ca^{2+}-independent low-affinity sites by washing with HBS/Ca/DS; these low-affinity sites do not mediate Lf internalization [8]. After being shifted to 37 °C, the preloaded cells rapidly internalized ^{125}I-Lf protein and Lf-bound ^{59}Fe within 6 min amounting to 81.5 and 82.5% respectively of ^{125}I-Lf and ^{59}Fe prebound at 4 °C (Figure 3a). First-order rate analyses of these internalization data revealed that hepatocytes accumulated ^{125}I-Lf protein and Lf-bound ^{59}Fe at statistically equal rates (Figure 3b; $P = 0.139$; ^{125}I-Lf: $k_{in} = 0.276$ min^{-1}, $r = 0.96$, $t_{1/2} = 2.51$ min; ^{59}Fe: $k_{in} = 0.303$ min^{-1}, $r = 0.96$, $t_{1/2} = 2.29$ min).

Hepatocytes hydrolyse internalized ^{125}I-Lf protein, regardless of its iron status, and release degradation products (e.g. ^{125}I-Tyr) in an acid-soluble form [9]. One might expect under these

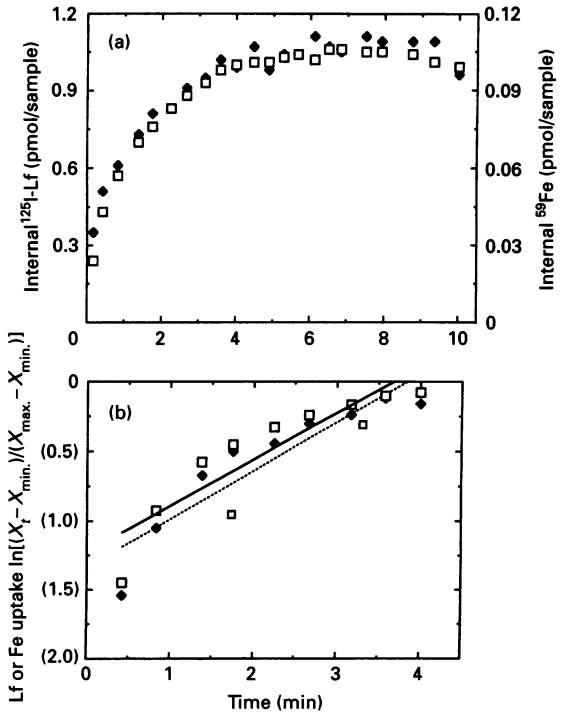

Figure 3 Determination of endocytic rate constants for uptake of prebound Lf–iron

(**a**) Cells were prebound with ^{125}I-^{59}Fe-Lf (5 μg/ml; ^{59}Fe/Lf molar ratio = 0.14) at 4 °C for 90 min and then washed in the presence of dextran sulphate to remove unbound Lf and Lf bound to Ca^{2+}-independent binding sites [9]. They were then incubated at 37 °C, and samples (10^6 cells) were assayed periodically for internalized radioactivity as described in the Experimental section. □, ^{125}I-Lf; ◆, ^{59}Fe. (**b**) Data were subjected to first-order rate analysis as described in the legend to Figure 1 to determine internalization rate constants. ^{125}I-Lf: $k_{in} = 0.276$ min^{-1}, $t_{1/2} = 2.5$ min, $r = 0.96$; ^{59}Fe: $k_{in} = 0.303$ min^{-1}, $t_{1/2} = 2.3$ min, $r = 0.96$. □, Lf; ◆, Fe. Parenthetic values are negative numbers.

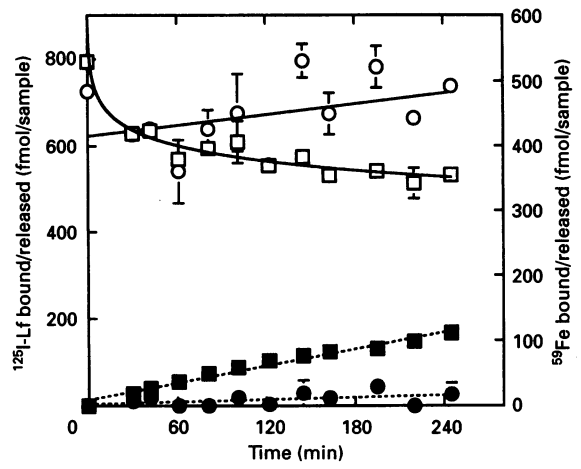

Figure 4 Kinetics of release of degradation products from hepatocytes after internalization of ^{125}I-^{59}Fe-Lf

Cells were prebound with ^{125}I-^{59}Fe-Lf (5 μg/ml; ^{59}Fe/Lf molar ratio = 0.44) at 4 °C for 90 min and then washed in the presence of dextran sulphate to remove unbound Lf and Lf bound to Ca^{2+}-independent binding sites [9], then incubated at 37 °C. At the designated times, 1 ml samples (1.5×10^6 cells) were chilled on ice and centrifuged to pellet cells which were assayed for associated radioactivity as described in the Experimental section. Protein in the cell media was precipitated by mixing 0.5 ml of the cell-free supernatants with 1.0 ml of ice-cold 10% (w/v) phosphotungstic acid in 2 M HCl on ice for ⩾ 15 min; this treatment precipitates ⩾ 96% of intact ^{125}I-Lf [9]. Acid-precipitated material was pelleted by centrifugation (4 °C; 3000 *g*; 7 min), and the supernatants were assayed for acid-soluble radioactivity. Symbols and error bars represent means ± ranges of duplicate samples. Best-fit isotherms were calculated according to the equations $y = -122.1\log(x) + 818.3$ (□, ^{125}I-Lf cell-associated), $y = 0.645(x) + 13.9$ (■, ^{125}I acid-soluble products), $y = 0.274(x) + 415.6$ (○, ^{59}Fe cell-associated) and $y = 0.057(x) + 2.7$ (●, ^{59}Fe released).

conditions that hepatocytes would retain Lf-delivered iron after Lf degradation. To test for this, hepatocytes prebound with ^{125}I-^{59}Fe-Lf at 4 °C were incubated at 37 °C and monitored for retention and release of Lf protein and Lf-bound iron (Figure 4). As expected, cell-associated ^{125}I-Lf protein decreased progressively throughout the time course, and acid-soluble ^{125}I-labelled degradation products were recovered in the medium; less than 3% of the radioactivity in the medium was acid-precipitable. In contrast, hepatocytes retained virtually all the Lf-bound ^{59}Fe during the 37 °C incubation, and little or no ^{59}Fe was detected in the medium. These results indicate that hepatocytes retained Lf-delivered Fe while degrading internalized Lf protein.

Modulation of ^{59}Fe uptake from Lf

The foregoing results show that hepatocytes internalized ^{125}I-Lf and Lf-bound ^{59}Fe at similar rates and to similar extents. There are two possible mechanisms for hepatocytes to acquire iron from Lf. One involves the endocytic uptake of iron complexed with Lf; the other involves the liberation of iron from Lf followed by facilitated or active transport of Fe^{2+} or Fe^{3+} into the cytoplasm at the plasma membrane. Many cells can transport iron directly into the cytoplasm at the plasma membrane [18–21]. For example, receptor-mediated endocytosis does not completely account for all the iron uptake by hepatocytes from Tf [3]. Some evidence suggests that hepatocytes liberate iron from Tf at the plasma membrane and transport it directly into the cytoplasm [22–24], although the specific details about this mechanism are somewhat controversial [25]. To understand the mechanism by which Lf donates its iron to hepatocytes, I examined the effects of iron chelators, Tf and endocytic inhibitors on Lf-dependent iron uptake by hepatocytes.

Tf does not compete with Lf for binding and endocytosis of Lf protein by hepatocytes [8,9], although Lf can partially inhibit hepatocyte iron uptake from Tf presumably by competing with Tf for binding to cell-surface proteoglycans [26]. It is not known, however, whether iron associated with transferrin and Lf follow common or unique entry routes into hepatocytes. For instance, if hepatocytes accumulate iron from Tf and Lf by 'non-transferrin-bound iron' transport [18], then iron-bound forms of the two proteins should compete with each other for iron uptake. To test this possibility, I determined whether large molar excesses of Tf altered hepatocyte uptake of ^{59}Fe-Lf and ^{125}I-Lf (Table 2). As expected, excess Lf diminished hepatocyte binding of ^{125}I-Lf and ^{59}Fe-Lf by 91 and 84% respectively. Excess diferric Tf reduced uptake of ^{125}I-Lf protein by hepatocytes by 20% ($P < 0.02$) and reduced uptake of Lf-bound ^{59}Fe by 33% ($P < 0.005$). These results indicated that Lf and Tf proteins for the most part did not compete with each other for binding to cells, but if present at high molar excess, diferric Tf could reduce hepatocyte accumulation of Lf-bound ^{59}Fe to a limited extent. Nonetheless, these data suggest that the iron-delivery pathways targeted by Lf and Tf are largely independent of one another or at least non-competitive at the ligand concentrations employed.

Isolated rat hepatocytes internalize macromolecules by clathrin-dependent and -independent pathways [27–29], the for-

Table 2 Effect of excess molar diferric Tf on Lf–iron endocytosis

Hepatocytes were incubated at 37 °C with ^{125}I-Lf or ^{59}Fe-Lf (2 μg/ml; ^{59}Fe/Lf molar ratio = 1.4) for 1 h in the presence or absence of a 100-fold molar excess of iron-saturated Lf (holo-Lf) or iron-saturated Tf (holo-Tf). Samples (2×10^6 cells) were assayed for total bound and intracellular radioactivity as described in the Experimental section. Values represent means ± ranges of duplicate samples.

	Ligand bound (pmol/sample)			
	^{125}I-Lf		^{59}Fe	
Additions	Total	Internal	Total	Internal
None	9.53 ± 1.33	9.65 ± 0.26	8.36 ± 0.12	8.57 ± 0.02
Holo-Lf	0.84 ± 0.01	0.73 ± 0.04	1.31 ± 0.04	1.14 ± 0.01
Holo-Tf	7.93 ± 0.62	7.76 ± 0.29	6.41 ± 0.09	5.77 ± 0.01

Table 3 Effect of Fe^{3+} chelator DFO and hyperosmolality on Lf–iron endocytosis

Hepatocytes were preincubated at 37 °C for 10 min with or without 0.25 M sucrose in standard BME/BSA or 4 mM DFO, then supplemented with either ^{125}I-Lf or ^{59}Fe-Lf (30 μg/ml) and incubated for an additional 45 min at 37 °C. Samples (2×10^6 cells) were assayed for total bound and intracellular radioactivity as described in the Experimental section. Values represent means ± ranges of duplicate samples.

	Ligand bound (pmol/sample)			
	^{125}I-Lf		^{59}Fe	
Additions	Total	Internal	Total	Internal
None	8.82 ± 1.10	7.53 ± 0.58	5.87 ± 0.12	4.36 ± 0.25
Sucrose	1.05 ± 0.13	0.58 ± 0.03	0.94 ± 0.05	0.53 ± 0.02
DFO	8.59 ± 0.13	6.70 ± 0.06	4.61 ± 0.33	4.62 ± 0.45

Table 4 Effect of the Fe^{2+} chelator BPS on Lf–iron endocytosis

Hepatocytes were incubated with ^{125}I-^{59}Fe-Lf (2 μg/ml) in the presence or absence of excess Lf (200 μg/ml) or BPS at 37 °C for 1 h. Samples (10^6 cells) were assayed for total bound and internalized radioactivity as described in the Experimental section. Values represent means ± ranges of duplicate samples. ^{59}Fe/Lf stoichiometry = 0.39.

	Ligand bound (pmol/mg of protein)			
	^{125}I-Lf		^{59}Fe	
Additions	Total	Internal	Total	Internal
None	2.22 ± 0.05	1.98 ± 0.04	1.05 ± 0.04	0.84 ± 0.11
Holo-Lf	0.42 ± 0.01	0.39 ± 0.04	0.21 ± 0.01	0.15 ± 0.01
BPS (25 μM)	2.78 ± 0.04	2.45 ± 0.1	1.26 ± 0.01	1.10 ± 0.10
BPS (250 μM)	3.05 ± 0.20	2.69 ± 0.03	1.36 ± 0.07	1.20 ± 0.02

mer of which can be selectively inhibited by hyperosmolality [28,30,31]. Moreover, hyperosmotic medium blocks endocytosis of Lf protein by hepatocytes [9], but does not inhibit binding of Lf to hepatocytes when assayed at 4 °C [8]. I examined therefore whether uptake of Lf-bound ^{59}Fe by hepatocytes required an intact clathrin-coated-pit endocytic pathway (Table 3). Hyperosmotic medium (~ 500 mmol/kg; sucrose as osmolite) reduced the internalization of Lf-bound ^{59}Fe and ^{125}I-Lf protein by 88 and 92% respectively compared with unmodified medium (~ 290 mmol/kg). Although hyperosmotic medium reduced the amount of ^{125}I-Lf protein and ^{59}Fe bound to the surface (total − internal), ~ 78% of the reduction in ^{125}I-Lf protein and ^{59}Fe accumulated by hepatocytes was due to reduced internalization. Because cells in hyperosmotic medium internalized a low amount of ^{125}I-Lf protein, it is likely that the reduction in ^{125}I-Lf bound to hepatocyte surfaces is due to entrapment of some Lf-binding sites along their intracellular itinerary during recycling. Taken together, these data suggested that hepatocytes acquired ^{59}Fe from Lf via a clathrin-dependent endocytic pathway and ruled out the possibility that cells internalized Lf-bound ^{59}Fe by a non-specific fluid-phase pathway.

Fe^{2+} and Fe^{3+} chelators block the uptake of ^{59}Fe from Tf in hepatocytes but not in reticulocytes [23,24]. This supports the hypothesis that hepatocytes can release iron from Tf at the plasma membrane, possibly via NADH-dependent reduction of Fe^{3+} to Fe^{2+}, then transport it to the cytoplasm. By this scenario, iron chelators absorb released iron before cellular import. Reticulocytes, on the other hand, acquire iron from Tf after endocytosis of Tf–iron complexes. In addition, peritoneal macrophages acquire iron from Lf by a process that is sensitive to iron chelators (B. E. Britigan, personal communication). To determine whether Lf-bound iron is released from Lf before incorporation by cells, hepatocytes were assayed for binding and internalization of ^{59}Fe-Lf and ^{125}I-Lf incubated in the presence and absence of the Fe^{3+} chelator DFO (Table 3) or the Fe^{2+} chelator bathophenanthroline disulphonate (BPS) (Table 4). DFO had little or no affect on accumulation of ^{125}I-Lf protein or ^{59}Fe-Lf by hepatocytes ($P < 0.05$). On the other hand, BPS treatment significantly enhanced both ^{125}I-Lf protein and Lf-bound ^{59}Fe binding and uptake (Table 4) by ~ 20% ($P < 0.05$). There was no significant difference in endocytosis of ^{125}I-Lf protein and Lf-bound ^{59}Fe observed between the two levels of BPS tested ($P < 0.38$). These data suggest therefore that Lf does not release its bound iron before internalization.

DISCUSSION

In this report, I investigated the acquisition of iron from Lf by isolated rat hepatocytes and have chronicled the following novel findings. (1) Hepatocytes vigorously internalized iron from Lf at rates and extents commensurate with that of the vesicular uptake of Lf protein. Hepatocytes degraded internalized Lf protein but retained Lf-delivered iron. (2) Hyperosmotic disruption of clathrin assembly at the plasma membrane blocked hepatocyte uptake of Lf-bound ^{59}Fe as well as the endocytosis Lf protein. (3) Large molar excesses of diferric Tf competed poorly with ^{59}Fe-Lf or ^{125}I-Lf for binding and endocytosis by hepatocytes. (4) The iron chelators DFO and BPS, which block hepatocyte uptake of iron from Tf [24], had no effect (DFO) or slightly enhanced (BPS) the uptake of ^{125}I-Lf protein and Lf-bound ^{59}Fe by hepatocytes. Taken together, these data strongly suggest that hepatocytes take up iron from Lf by clathrin-dependent endocytosis of Lf–iron complexes rather than by active transport of Fe^{3+} or Fe^{2+} released from Lf at the plasma membrane.

Virtually all studies examining Lf interaction with liver cells employ a heterologous system of human, bovine or murine Lf with rat hepatocytes. Notably, Lfs show some species-specificity for binding cells [9,32]. Work in my laboratory has identified Lf in rat neutrophil lysates (D. J. Bennatt and D. D. McAbee, unpublished work), but quantities of rat Lf sufficient for biochemical studies are not available at present. Bovine Lf appears to be a reasonable substitute because it binds rat hepatocytes with much higher affinity (K_d ~ 20 nM [8]) than does human Lf

($K_d \sim 10\ \mu M$ [7]). Importantly, the steady-state concentration of Lf in blood is ~ 20 nM [4]. This type of high-affinity interaction is required if the Ca^{2+}-dependent Lf-binding sites are to maintain physiological Lf levels in blood and reflects a high degree of biological specificity.

Lf binds to macrophages [5,33–35], monocytes [36–39], T- and B-lymphocytes [36,40,41], intestinal mucosae and intestinal cell lines [42–44], hepatocytes [7,8] and platelets [45], but the extent to which these different cells internalize Lf protein and its bound iron varies dramatically. Thus the ability of Lf to function as an iron-transport protein has been unclear and somewhat controversial. Findings presented here (Table 1, Figures 1 and 2) and elsewhere [8,9], however, indicate that isolated hepatocytes internalize Lf protein at a rate of ~ 5000 mol·cell^{-1}·s^{-1} by a clathrin-dependent endocytic pathway, regardless of the iron content of Lf. In addition, hepatocytes accumulate Lf-bound iron at rates similar to that for Lf protein (Figures 1 and 2). It follows therefore that hepatocytes could accumulate ~ 10000 mol of iron·cell^{-1}·s^{-1} from Lf depending on the degree of iron saturation. By comparison, rat hepatocytes take up iron from Tf at maximal rates between 1500 and 17000 mol·cell^{-1}·s^{-1} [24,46]. Hepatocytes also incorporate iron from extracellular ferritin at maximal rates between 2600 and 80000 mol·cell^{-1}·s^{-1} depending on the size and iron content of ferritin cores internalized [2,47]. Findings presented here therefore suggest that hepatocyte accumulation of iron from Lf is comparable with that from Tf and ferritin, which are iron-transport/scavenging proteins implicated in hepatic iron retrieval during turnover of senescent erythrocytes [47]. The participation of Lf as an iron scavenger in routing iron from the reticuloendothelial system to the liver, however, remains to be defined fully.

An important issue is whether or not iron must remain bound to Lf for it to be internalized by cells. Because a variety of cells including hepatocytes can transport free iron directly into the cytoplasm at the plasma membrane [48,49], it is possible that iron is released from Lf at the cell surface and subsequently delivered to the cell by a non-vesicular transport pathway. Hepatocytes may take up iron from Tf by both a non-vesicular transport pathway and a Tf-receptor-dependent endocytic pathway [1,22,24,50]. Several lines of evidence presented here strongly suggest that hepatocyte uptake of iron from Lf requires endocytosis of Lf–iron complexes. (i) The rates and extents by which hepatocytes acquired ^{59}Fe from Lf were similar to their endocytic accumulation of Lf protein (Figures 1–3). (ii) Diferric Tf competed poorly with uptake of ^{59}Fe and ^{125}I-Lf protein only when present at high molar excess (Table 2), indicating that the iron-delivery mechanisms used by Lf and Tf are largely independent of each other. It is likely that at the Tf/Lf ratio used in this experiment Tf competed with Lf for binding to cell-surface proteoglycan [26] which may constitute in part the low-affinity Ca^{2+}-independent Lf-binding sites on hepatocytes [8,9]. Tf inhibited, albeit at low levels, Lf protein and ^{59}Fe uptake by similar extents (Table 2), supporting the conclusion that hepatocytes acquire iron from Lf by endocytosis of Lf–iron complexes. (iii) Hyperosmotic disruption of the clathrin-coated-pit pathway blocked uptake of both Lf protein and Lf-bound iron (Table 3), consistently with the notion that entry of Lf-bound iron into hepatocytes occurs by endocytosis. (iv) Fe^{2+} and Fe^{3+} chelators either had no effect or enhanced Lf protein and Lf-bound iron uptake by hepatocytes (Tables 3 and 4). Because these chelators were present in large molar excess over Lf, these findings confirm that Lf does not release its bound iron before internalization by hepatocytes. Notably, both DFO and BPS at concentrations similar to those used here block hepatocyte uptake of iron from Tf [23,24]. Lf-bound iron is transported into and across HT-29cl.19A human intestinal cells complexed with Lf [44]. Uptake of iron from Lf by peritoneal macrophages, however, is inhibited by BPS (B. E. Britigan, personal communication) suggesting that these cells either release iron from Lf at the plasma membrane or partition Lf-donated iron into a cellular pool readily mobilized by BPS. Others have found that monocytes, which bind large amounts of Lf, exhibited modest degrees of uptake of Lf protein and Lf-bound iron [37,39,40]. These findings suggest that the mechanism by which Lf donates its iron to cells may vary depending on the nature of the Lf-binding site present on different cells. At present, my laboratory is investigating the intracellular trafficking of Lf protein and Lf-bound iron within hepatocyte endocytic compartments.

Finally, results presented here suggest the possibility that hepatocytes may vary Lf endocytosis and Lf-dependent iron uptake as a function of the iron status of the cell. Cells incubated with diferric Tf showed minor reductions in iron uptake from Lf (Table 2). On the other hand, cells incubated with BPS significantly increased endocytosis of Lf protein and Lf-bound iron (Table 4). This suggests the possibility that the amount of iron present in the non-haem iron pools within hepatocytes may regulate the Lf endocytic pathway. Studies are at present underway to investigate the effect iron status has on hepatocyte iron uptake from Lf.

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REFERENCES

1 Morgan, E. H. and Baker, E. (1986) Fed. Proc. Fed. Am. Soc. Exp. Biol. **45**, 2810–2816
2 Sibille, J.-C., Kondo, H. and Aisen, P. (1988) Hepatology **8**, 296–301
3 Thorstensen, K. and Romslo, I. (1990) Biochem. J. **271**, 1–10
4 Bennett, R. M. and Kokocinski, T. (1979) Clin. Sci. **57**, 453–460
5 Imber, M. J. and Pizzo, S. V. (1983) Biochem. J. **212**, 249–257
6 Regoeczi, E., Chindemi, P. A., Debanne, M. T. and Prieels, J. P. (1985) Am. J. Physiol. **248**, G8–G14
7 Ziere, G. J., Vandijk, M. C. M., Bijsterbosch, M. K. and Vanberkel, T. J. C. (1992) J. Biol. Chem. **267**, 11229–11235
8 McAbee, D. D. and Esbensen, K. (1991) J. Biol. Chem. **266**, 23624–23631
9 McAbee, D. D., Nowatzke, W., Oehler, C. et al. (1993) Biochemistry **32**, 13749–13760
10 Seglen, P. O. (1973) Exp. Cell Res. **82**, 391–398
11 Oka, J. A. and Weigel, P. H. (1987) J. Cell. Physiol. **133**, 243–252
12 Masson, P. L. and Heremans, J. F. (1968) Eur. J. Biochem. **6**, 579–584
13 Schryvers, A. B. and Morris, L. J. (1988) Infect. Immun. **56**, 1144–1149
14 Brown, E. M. and Parry, R. M. (1974) Biochemistry **13**, 4560–4565
15 Hashizume, S., Kuroda, K. and Murakami, H. (1987) Methods Enzymol. **147**, 302–314
16 Fraker, P. J. and Speck, J. C. (1978) Biochem. Biophys. Res. Commun. **80**, 849–857
17 Smith, P. K., Krohn, R. I., Hermanson, G. T. et al. (1985) Anal. Biochem. **150**, 76–85
18 Sturrock, A., Alexander, J., Lamb, J., Craven, C. M. and Kaplan, J. (1990) J. Biol. Chem. **265**, 3139–3145
19 Kaplan, J., Jordan, I. and Sturrock, A. (1991) J. Biol. Chem. **266**, 2997–3004
20 Randell, E. W., Parkes, J. G., Olivieri, N. F. and Templeton, D. M. (1994) J. Biol. Chem. **269**, 16046–16053
21 Britigan, B. E., Serody, J. S., Hayek, M. B., Charniga, L. M. and Cohen, M. S. (1991) J. Immunol. **147**, 4271–4277
22 Wright, T. L., Fitz, J. G. and Weisiger, R. A. (1988) J. Biol. Chem. **263**, 1842–1847
23 Thorstensen, K. and Romslo, I. (1988) J. Biol. Chem. **263**, 8844–8850
24 Thorstensen, K. (1988) J. Biol. Chem. **263**, 16837–16841
25 Thorstensen, K. and Aisen, P. (1990) Biochim. Biophys. Acta **1052**, 29–35
26 Hu, W.-L., Regoeczi, E., Chindemi, P. A. and Bolyos, M. (1993) Am. J. Physiol. **264**, G112–G117

27 England, I. G., Naess, L., Blomhoff, R. and Berg, T. (1986) Biochem. Pharmacol. **35**, 201–208
28 Oka, J. A., Christensen, M. D. and Weigel, P. H. (1989) J. Biol. Chem. **264**, 12016–12024
29 Thirion, J. and Wattiaux, R. (1988) Biochem Biophys. Res. Commun. **152**, 1275–1281
30 Daukas, G. and Zigmond, S. H. (1985) J. Biol. Chem. **101**, 1673–1679
31 Heuser, J. E. and Anderson, R. G. W. (1989) J. Cell Biol. **108**, 389–400
32 Kawakami, H. and Lonnerdal, B. (1991) Am. J. Physiol. **261**, G841–G846
33 Van Snick, J. L. and Masson, P. L. (1976) J. Exp. Med. **144**, 1568–1580
34 Van Snick, J. L., Markowetz, B. and Masson, P. L. (1977) J. Exp. Med. **146**, 817–827
35 Campbell, E. J. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 6941–6945
36 Bennett, R. M. and Davis, J. (1981) J. Immunol. **127**, 1211–1216
37 Birgens, H. S., Kristensen, L. O., Borregaard, N., Karle, H. and Hansen, N. E. (1988) Eur. J. Haematol. **41**, 52–57
38 Oria, R., Alvarez-Hernandez, X., Liceaga, J. and Brock, J. H. (1988) Biochem. J. **252**, 221–225
39 Ismail, M. and Brock, J. H. (1993) J. Biol. Chem. **268**, 21618–21625
40 Birgens, H. S., Hansen, N. E., Karle, H. and Kristensen, L. O. (1983) Br. J. Haematol. **54**, 383–391
41 Mazurier, J., LeGrand, D., Hu, W. L., Montreuil, J. and Spik, G. (1989) Eur. J. Biochem. **179**, 481–487
42 Davidson, L. A. and Lonnerdal, B. (1988) Am. J. Pathol. **254**, G580–G585
43 Hu, W.-L., Mazurier, J., Sawatzki, G., Montreuil, J. and Spik, G. (1988) Biochem. J. **249**, 435–441
44 Mikogami, T., Heyman, M., Spik, G. and Desjeux, J. F. (1994) Am. J. Physiol. **267**, G308–G315
45 Leveugle, B., Mazurier, J., Legrand, D., Mazurier, C., Montreuil, J. and Spik, G. (1993) Eur. J. Biochem. **213**, 1205–1211
46 Trinder, D., Batey, R. G., Morgan, E. H. and Baker, E. (1990) Am. J. Physiol. **259**, G611–G617
47 Osterloh, K. and Aisen, P. (1989) Biochim. Biophys. Acta **1011**, 40–45
48 Kaplan, J., Jordan, I. and Sturrock, A. (1991) J. Biol. Chem. **266**, 2997–3004
49 Quail, E. A. and Morgan, E. H. (1994) J. Cell. Physiol. **159**, 238–244
50 Sibille, J.-C., Octave, J.-N., Schneider, Y.-J., Trouet, A. and Crichton, R. (1986) Eur. J. Biochem. **155**, 47–55

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