Caco-2 Cell Ferritin Formation Predicts Nonradiolabeled Food Iron Availability in an In Vitro Digestion/Caco-2 Cell Culture Model[1]

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ABSTRACT We have adapted an in vitro digestion/Caco-2 cell model to assess Fe availability from foods, by using ferritin formation by Caco-2 cells as an indicator of Fe uptake. Ferritin formation by Caco-2 cells occurs in response to Fe uptake at concentrations of available Fe greater than that of the culture media to which the cells have been adapted. This methodology circumvents the need for using radioactive Fe and thus eliminates the costs and controversies associated with food radiolabeling. To validate this method, we measured ferritin formation in Caco-2 cells exposed to digests containing Fe of relatively high and low availability. Our objective was to determine if ferritin formation would be proportional to Fe uptake and sufficiently sensitive to be an indicator of Fe availability from food digests. Our model uses established in vitro digestion techniques coupled with uptake of Fe by Caco-2 cell monolayers. Measurement of cell ferritin was done by a commercially available RIA. Higher ferritin formation was observed in cells exposed to digests containing $FeSO_4$ plus ascorbic acid vs, digests containing $FeSO_4$ plus citric acid. Additional comparisons of Fe availability from digests of beef, fish, corn and green beans yielded results that demonstrate higher Fe availability (i.e., greater ferritin formation) from beef and fish digests than from digests of corn and green beans. Overall, the results document the promotional effects of ascorbic acid and animal tissue on Fe uptake as measured indirectly by ferritin formation. The results of this study indicate that ferritin formation by Caco-2 cell monolayers is highly sensitive and accurately measures food Fe availability in this in vitro system. J. Nutr. 128: 1555–1561, 1998.

KEY WORDS: • *ferritin* • *Caco-2* • *in vitro digestion* • *iron availability*

The Caco-2 cell line is a useful model for studies of intestinal human iron uptake (Alvarez-Hernandez et al. 1991, Han et al. 1994 and 1995). We have utilized the Caco-2 cell line in conjunction with in vitro digestion techniques and have developed a model whereby foods undergo simulated peptic digestion followed by intestinal digestion in the presence of Caco-2 cell monolayers (**Fig. 1**). This model measures iron solubility in addition to providing a measure of uptake via a living component, the Caco-2 cell monolayer, and is a great advancement over the use of in vitro digestion alone, which measures only iron solubility and therefore is not a complete measure of Fe availability (Glahn et al. 1998, Miller and Berner 1989).

The conditions of this model have been designed to simulate the gastrointestinal environment while still maintaining a rapid and inexpensive system. This model system is unique among applications of Caco-2 cells and in vitro digestion techniques in that it allows uptake to occur simultaneously with food digestion under pH conditions similar to those found along the absorptive surface of the intestinal tract (Berne and Levy 1993, Guyton and Hall 1996). Furthermore, the addition of the human-derived component, i.e., Caco-2 cells, transforms this model system into a unique tool, capable of conducting experiments that might not be feasible or practical to conduct in vivo. When used as a prelude to human trials, this model may enable improved design and productivity of the more expensive human experiment. Anticipated and ongoing applications of this model include food product development of infant formula and cereals, screening of plant cultivars for improved iron availability, development of improved iron supplements and studies of the precise factors in digests of animal tissue that promote iron uptake.

Previous applications of this model required extrinsic radiolabeling of the food iron (Glahn et al. 1996 and 1998). Those studies were primarily validation trials designed to document the enhancing effects of animal tissue and ascorbic acid on iron uptake, and to illustrate how this model system could be applied to the development of foods with improved iron availability. The authors of this manuscript recognize that extrinsic radiolabeling of food iron remains controversial for some conditions encountered in foods (Consaul and Lee 1984, Van Campen 1983). However, for food products such as infant formula, where essentially all of the iron is added as $FeSO_4$, extrinsic radiolabeling should be appropriate (Glahn et al. 1998). Intrinsic radiolabeling, an alternative means of tracking

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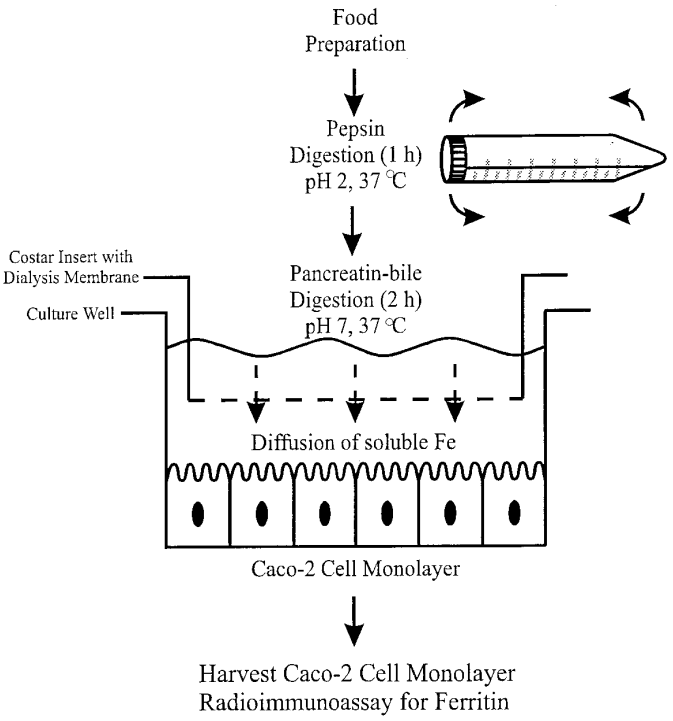

FIGURE 1 Diagram of in vitro digestion/Caco-2 cell culture model.

iron absorption commonly used for plant foods, is relatively expensive, time-consuming and requires a facility and technical staff to support the growth of radiolabeled plant materials. In addition, it may be difficult to incorporate sufficient radioactivity into the food of study, particularly for use with in vitro systems (Glahn, unpublished observations). In light of the above information, an in vitro model that does not require radiolabeling of the food iron would represent a significant advantage in both time, cost and ease of use. It would enable measurement of food iron availability in foods directly from the producer or supermarket shelf and would eliminate concerns of adequate radiolabeling of food iron from a complete meal.

Because cellular iron is stored as ferritin, we hypothesized that available iron would induce greater ferritin formation in Caco-2 cells than less available forms (Gangloff et al. 1996). If so, ferritin formation could be used as an indicator of cell iron uptake, thus eliminating the need for radiolabeling of food iron in availability assays. The objective of this study was to determine if the above relationship exists and if so, to define, demonstrate and thereby validate the use of Caco-2 cell ferritin formation as a method for measuring food iron availability.

MATERIALS AND METHODS

Chemicals, enzymes and hormones. Unless otherwise stated, all chemicals, enzymes and hormones were purchased from Sigma Chemical (St. Louis, MO).

Cell culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17, and used in experiments at passage 25–33. Cells were seeded at a density of 50,000 cells/cm^2 in collagen-treated 6-well plates (6-well cell culture cluster dishes, Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) with 10% v/v fetal calf serum (GIBCO), 25 mmol/L HEPES and 1% antibiotic antimycotic solution (GIBCO). The cells were maintained at 37°C in an incubator with a 5% CO_2/ 95% air atmosphere at constant humidity; the medium was changed every 2 d. The cells were used in the iron uptake experiments at 13 d postseeding. Under these conditions, the amount of cell protein measured in each well was highly consistent from well to well within each culture plate.

In vitro digestion. Porcine pepsin (800–2500 units/mg protein), pancreatin (activity, 4 × USP specifications) and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts) were purchased from Sigma Chemical. Further preparation of the pepsin, pancreatin and bile extract was performed as follows. Shortly before use, 0.2 g pepsin was dissolved in 5 mL of 0.1 mol/L HCl, added to 2.5 g of Chelex-100 (Bio-Rad Laboratories, Hercules, CA) and shaken on a tabletop shaker for 30 min. The pepsin solution with Chelex was then poured into a 1.6-cm diameter filtration column to filter out the Chelex from the pepsin solution. An additional 5 mL of 0.1 mol/L HCl was added to the column and the filtrate collected into the pepsin solution. The final total volume of the eluted pepsin solution was 8 mL.

For the intestinal digestion, 0.05 g pancreatin and 0.3 g bile extract were dissolved in 25 mL of 0.1 mol/L $NaHCO_3$. Chelex-100 (12.5 g) was added and the resulting mixture was shaken for 30 min on a tabletop shaker. The mixture was then poured into a 1.6-cm diameter filtration column to filter out the Chelex. An additional 10 mL of 0.1 mol/L $NaHCO_3$ was added to the column and the filtrate collected into the pancreatin/bile solution. The final total volume of the pancreatin/bile solution was 27 mL. Treatment of the pepsin and pancreatin-bile solutions via the methods described above did not affect the activity of the enzymes.

Peptic and intestinal digestions were conducted on a rocking platform shaker (Reliable Scientific, Hernando, MS) in an incubator at 37°C with a 5% CO_2/95% air atmosphere maintained at constant humidity. The intestinal digestion was carried out in the upper chamber of a two-chamber system in 6-well plates, with the cell monolayer attached to the bottom surface of the lower chamber (Fig. 1). The upper chamber was formed by fitting the bottom of an appropriately sized Transwell insert ring (gift from Costar) with a 15,000 molecular weight cut-off dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). The membranes were soaked in deionized water before use. The dialysis membrane was held in place with a silicone ring (Web Seal, Rochester, NY). After the dialysis membrane was fastened to the insert ring, the entire unit was sterilized in 70% ethanol and then kept in sterile water until use.

To start the peptic digestion, the pH of each sample was adjusted to pH 2.0 with 5.0 mol/L HCl. The sample was transferred to a 50-mL screw-cap culture tube, and 0.5 mL of the pepsin solution was added per 10 mL of sample. The tube was capped, placed horizontally and incubated for 60 min on the rocking shaker at rocking speed #7 (55 oscillations/min). For the intestinal digestion step, the pH of the sample (also referred to as the "digest") was raised to pH 6 by dropwise addition of 1 mol/L $NaHCO_3$. Then 2.5 mL of pancreatin-bile extract mixture was added per 10 mL of original sample. The pH was adjusted to pH 7 with NaOH, and the volume was brought to 15 mL with 120 mmol/L NaCl and 5 mmol/L KCl.

Preparation of the 6-well culture plates with cell monolayers. Immediately before the intestinal digestion period, the growth medium was removed from each culture well and the cell layer was washed twice with 37°C Minimum Essential Medium (MEM, GIBCO) at pH 7. This MEM was chosen because it contained no added Fe; upon formulation with the following ingredients, it was always found to contain <8 μg Fe/L. The MEM was supplemented with 10 mmol/L PIPES (piperazine-*N*,*N*′-bis-[2-ethanesulfonic acid]), 1% antibiotic-antimycotic solution (Sigma), hydrocortisone (4 mg/L), insulin (5 mg/L), selenium (5 μg/L), triiodothyronine (34 μg/L) and epidermal growth factor (20 μg/L). A fresh 1.0-mL aliquot of MEM covered the cells during the experiment. A sterilized insert ring, fitted with a dialysis membrane, was then inserted into the well, thus creating the two-chamber system. Then a 1.5-mL aliquot of the intestinal digest was pipetted into the upper chamber. The plate was covered and incubated on the rocking shaker at 6 oscillations/min for 120 min.

When the intestinal digestion was terminated, the insert ring and digest were removed. The solution in the bottom chamber was allowed to remain on the cell monolayer and an additional 1 mL of MEM was added to each well. The cell culture plate was then returned to the incubator for an additional 22 h, after which the cells were harvested for analysis.

Harvesting of Caco-2 cell monolayers for ferritin analysis and cell-associated Fe. Exactly 24 h after the start of the intestinal digestion period, the cell monolayers were harvested for various analyses. To harvest the cells, the medium covering the cells was removed and the cells washed once with a 2 mL volume of a "rinse" solution containing 140 mmol/L NaCl, 5 mmol/L KCl and 10 mmol PIPES, at pH 7. The "rinse" solution was then aspirated and a 2-mL volume of a freshly prepared "removal" solution was placed on the cell monolayer for 10 min. The "removal" solution consisted of the above rinse solution with an additional 5 mmol/L sodium hydrosulfite and 1 mmol/L bathophenanthrolene disulfonic acid (BPDS). In results from our laboratory (unpublished), we found that lowering the BPDS level in the removal solution to 1 mmol/L is as effective as the 5 mmol/L BPDS solution used previously (Glahn et al. 1995).

After the removal period, the removal solution was aspirated and the cell monolayer washed with a 2-mL volume of rinse solution. The rinse solution was then aspirated and 2 mL of deionized water was placed on each monolayer. The plates were then placed on a rack such that the bottom of each plate was in contact with the water of a benchtop sonicator, which was kept in a cold room at 4°C. The cells were sonicated for 15 min, scraped from the plate surface, harvested along with the 2 mL volume of water in each well and then stored at −20°C.

Measurement of soluble iron: use of companion plates without cells. For each experiment, two 6-well plates without Caco-2 cells were used as "companion" plates. These plates were treated identically to those with cells. The companion plates served to determine the amount of iron that passed into the bottom chamber under the conditions defined in this study. Because a large portion of the iron that passes into the bottom chamber may be taken up by the cells, companion plates were used for more accurate measurement of the amount of dialyzable iron. By using centrifugation, we found that virtually all of the iron that passed into the bottom chamber of this model system was soluble (Glahn, unpublished observations).

Experimental design. Experiments were replicated four to five times for each experimental protocol. Each experimental treatment was performed in duplicate for each replication of the experiment. The duplicates were averaged and this average value was the data point used in the statistical analysis. The position of each experimental treatment in the multiwell plate was different for each replication of the experiment. Replicates of each experiment were conducted on separate days. The exact number of replicates is noted in the figure legends.

The first and second series of experiments (Figs. 2 and 3, respectively) were designed to determine if ferritin formation by Caco-2 cells over a 24-h period was proportional to cell iron uptake. To do so, varying amounts of Fe (0–200 μmol/L) were added to the digest in the following forms: $FeSO_4$ with excess ascorbic acid (1 mmol/L) or $FeSO_4$ in the presence of citric acid at a 2:1 citrate to Fe ratio. Under the conditions used in this study, iron added as $FeSO_4$ in the presence of excess ascorbic acid should be highly available, whereas $FeSO_4$ in the presence of citrate should be less available (Alvarez-Hernandez et al. 1991). According to the hypothesis stated earlier, the more highly available forms of Fe should result in higher ferritin formation.

The final series of experiments (Fig. 4) compared samples of beef, corn, fish (haddock) and green beans. The beef and fish samples were from freeze-dried cooked samples described previously and contained 98.8 and 6.43 μg Fe/g sample, respectively (Glahn et al. 1996). The corn sample was from a generic cornmeal purchased at a local market. It contained 18.1 μg Fe/g of sample. The sample of green beans was purchased at a local supermarket in puréed form as supplied by Gerber Products (Fremont, MI) as stage 1 food for infants. The green bean sample contained 6.08 μg Fe/g of sample. Digests of the above foods contained 10 μg Fe from each food.

Analyses. All glassware used in the sample preparation and analyses was acid-washed. Caco-2 cell protein was measured on samples that had been solubilized in 0.5 mol/L NaOH, using a semimicro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories). A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-IRON II Ferritin Assay, RAMCO Laboratories, Houston, TX). A 10-μL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each ferritin measurement. Pilot studies had determined that centrifuging the Caco-2 cell sample before sampling was not necessary for accurate ferritin measurement. Analyses of the iron content of the experimental solutions, foods, digests and Caco-2 cell monolayers were conducted by using an inductively coupled plasma emission spectrometer (ICAP Model 61E Trace Analyzer, Thermo Jarrell Ash Corporation, Franklin, MA).

Statistics. Statistical analysis of the data was performed using the software package GraphPad Prism (GraphPad Software, San Diego, CA). Statistical analyses were conducted according to the methods of Motulsky (1995). Before analysis, data were log transformed to achieve equal variance. Because each replication of an experiment in our study was a paired comparison, a repeated measures ANOVA was performed with Tukey's post-hoc test to compare the various means of each series of experiments. Means were considered significantly different if P-values were ≤ 0.05.

RESULTS

Figure 2 summarizes the effect of increasing Fe in the digest (in the presence of 1 mmol/L ascorbic acid) on ferritin formation by Caco-2 cell monolayers. Digests were formulated to have 0, 10, 20, 50 and 100 μmol/L Fe added as $FeSO_4$. An additional digest containing only 1 mmol/L ascorbic acid was added to this series of experiments. The ascorbic acid digest served as a control to monitor the promotional effect of the ascorbic acid on the trace amounts of Fe contributed from the digestive enzymes and system components. Figure 2*A* represents the total Fe measured in a 1.5-mL aliquot of the digest that was placed in the upper chamber at the start of the intestinal digestion period. The amount of Fe measured in an aliquot that was added to the upper chamber was consistent with the expected Fe content of the digest (Fig. 2*A*). Increasing amounts of Fe in the digest resulted in corresponding increases in the amount of Fe that diffused into the bottom chamber (Fig. 2*B*). Figure 2*C* represents the amount of Fe present in the bottom chamber solution 24 h after the start of the intestinal digestion period. For this measurement, the digest formulated to contain 100 μmol/L Fe was significantly greater than the other digests, all of which exhibited Fe concentration similar to that of the blank digest. Presumably, the cells took up most of the Fe that diffused into the bottom chamber with the exception of the treatment containing 100 μmol/L Fe. Figure 2*D* illustrates the Fe associated with the cell monolayer as measured by inductively coupled plasma (ICP) emission spectroanalysis. Caco-2 cell Fe content corresponded to increased amounts of Fe in the digest. Ferritin formation increased with increasing digest Fe concentration (Fig. 2*E*). The digest containing only ascorbic acid and no added Fe exhibited a significant increase in ferritin formation relative to the blank digest, but was significantly less than that of the digest containing 10 μmol/L Fe. Also of interest in this figure is the lack of a significant difference in ferritin formation between the digests containing 50 and 100 μmol/L Fe. Plotting cell Fe content vs. cell ferritin formation resulted in a nonlinear correlation as shown in Figure 2*F*.

Ferritin formation from cells exposed to digests containing $FeSO_4$ in the presence of citric acid at a 2:1 molar ratio of citrate to Fe is summarized in **Figure 3**. As expected, the amount of Fe placed in the upper chamber increased proportionately with the calculated Fe concentration of the digest (Fig. 3*A*). Relative to the blank digest, the amount of Fe that diffused into the bottom chamber of plates with no cells present increased proportionately for digest Fe concentrations ≥50 μmol/L (Fig. 3*B*). Caco-2 cell ferritin formation increased consistently in response to digest Fe concentration (Fig. 3*C*). A graph of cell Fe content vs. cell ferritin formation exhibited a relatively linear relationship (Fig. 3*D*).

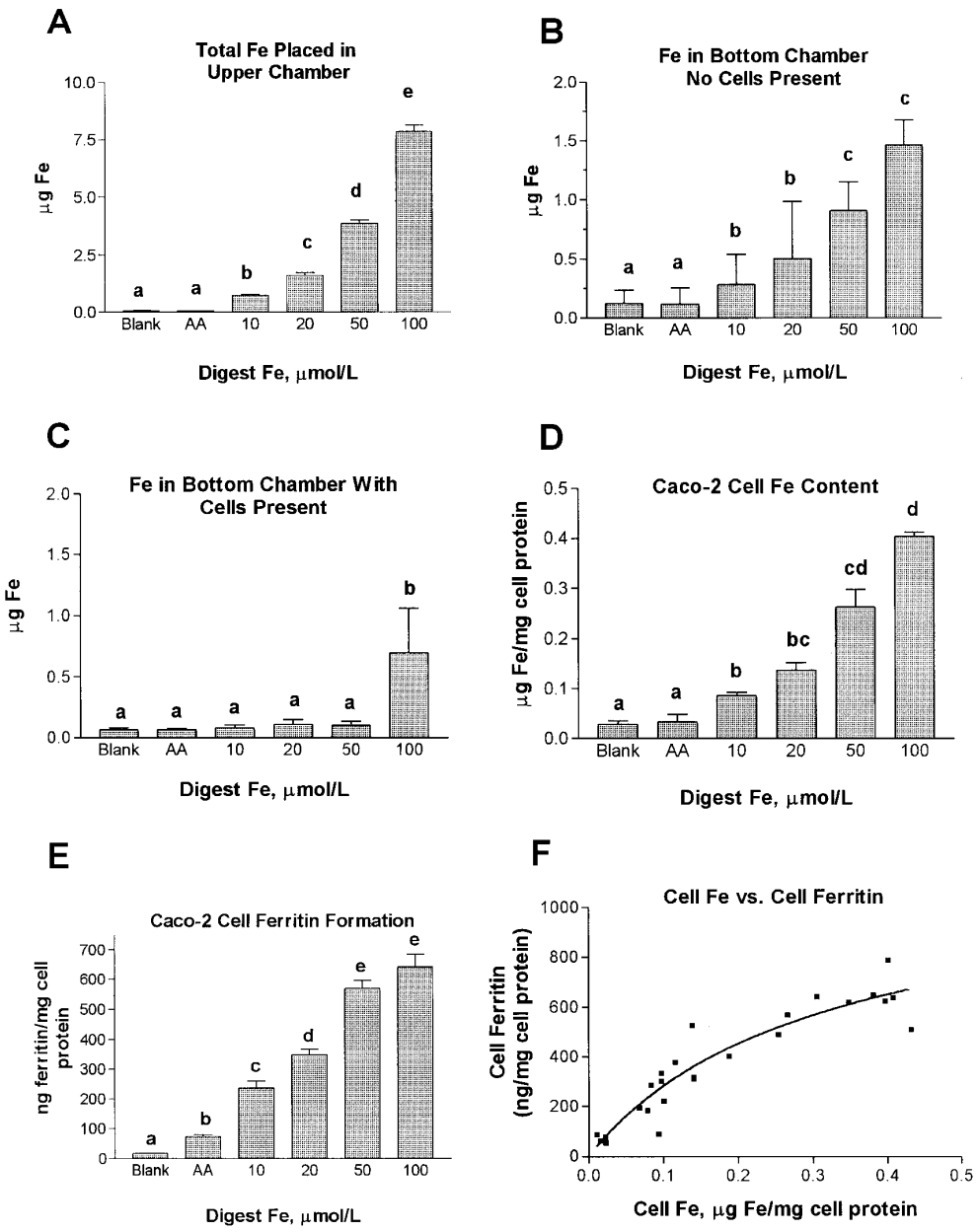

FIGURE 2 Measured variables for experiments comparing digests containing $FeSO_4$ (0–100 μmol/L) combined with ascorbic acid (AA) at a concentration of 1 mmol/L. Digest concentrations of $FeSO_4$ and AA represent values calculated from the formulation at the start of the intestinal digestion period. "Blank" indicates digest system components only (i.e., pepsin, pancreatin, bile extract), no added $FeSO_4$ or AA. Bars (mean ± SEM, $n = 5$) with no letters in common are significantly different ($P < 0.05$). Specific panels are defined as follows: (*A*) total amount of Fe measured in 1.5 mL of digest placed in the upper chamber at the start of the intestinal digestion period; (*B*) amount of Fe measured in the bottom chamber of "companion plates" (i.e., no cells present) immediately after the 2-h intestinal digestion period; (*C*) amount of Fe measured in the bottom chamber of wells with cells present. Samples collected 24 h after the start of the intestinal digestion period; (*D*) Caco-2 cell Fe content after harvest of the cells 24 h after the start of the intestinal digestion period; (*E*) Caco-2 cell ferritin formation as measured 24 h after the start of the intestinal digestion period; (*F*) plot of cell Fe content vs. cell ferritin measured 24 h after the start of the intestinal digestion period. Pearson $r = 0.91$.

It is important to note that Caco-2 cell ferritin formation from cells exposed to digests containing $FeSO_4$ plus ascorbic acid was more than twice that observed for $FeSO_4$ combined with citric acid (Figs. 2*E* and 3C).

Comparisons of Fe availability from digests of beef, corn, fish and green beans are summarized in **Figure 4**. As a positive control and reference point, a digest of $FeSO_4$ in the presence of 1 mmol/L ascorbic acid was used in this series of experiments. For these experiments, an appropriate amount of each food was used, which resulted in a contribution of 10 μg Fe by the intrinsic Fe of the food. Thus, the amount of food used in each digest was as follows: beef, 0.10 g; corn, 0.55 g; fish, 1.55 g; and green beans, 1.65 g. Figure 4*A* indicates that digest Fe concentrations were similar for all foods. Ferritin formation from digests containing fish was more than twice as much as that for beef, corn or green beans when expressed as ng ferritin/mg cell protein (Fig. 4*B*). Expressing ferritin formation as a percentage of the $FeSO_4$ plus ascorbic acid digest may also be useful and is shown in Figure 4C. Significant difference between the beef and fish digests was not observed for this calculation as a result of the relatively high variability of the fish digest. Calculations of ferritin formation per gram of food are presented in Figure 4*D*. Digests of beef yielded six to seven times the amount of ferritin formation per gram of food relative to the corn and fish digest, which were observed to be 3–4 times greater than the green bean digest.

DISCUSSION

Ferritin formation by intestinal epithelial cells occurs in response to iron that has been taken up but not transported across the basolateral surface (Beard et al. 1996). Previous studies of the relationship between Fe uptake and Caco-2 cell ferritin content demonstrated an inverse correlation between the rate of iron uptake and the "iron status" of the cell monolayer (Alvarez-Hernandez et al. 1991, Gangloff et al. 1996). That is, Caco-2 cell monolayers cultured in low Fe–containing media exhibited low ferritin content and a high rate of iron

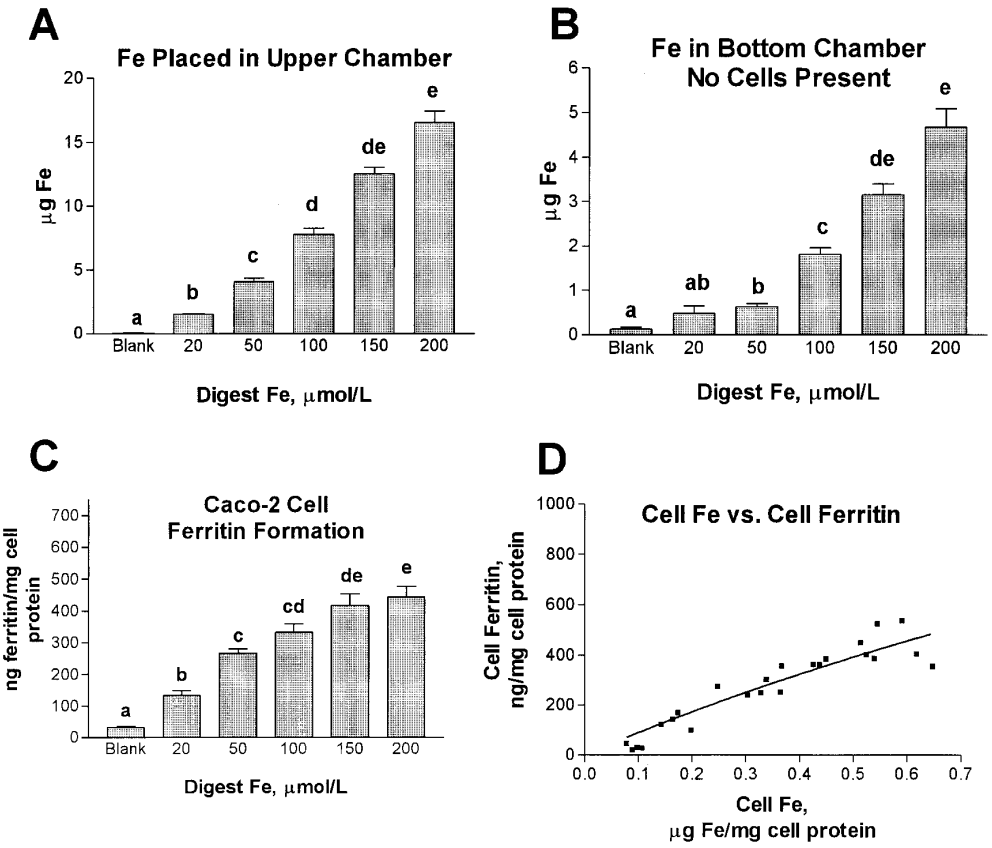

FIGURE 3 Measured variables for experiments comparing digests containing $FeSO_4$ (0–200 μmol/L) combined with citric acid at a 2:1 molar ratio of citric acid to Fe. Digest concentrations of $FeSO_4$ and citric acid represent values calculated from the formulation at the start of the intestinal digestion period. "Blank" indicates digest system components only (i.e., pepsin, pancreatin, bile extract), no added Fe or citric acid. Bars (mean ± SEM, $n = 4$) with no letters in common are significantly different ($P < 0.05$). Specific panels are defined as follows: (*A*) amount of Fe measured in 1.5 mL of digest placed in the upper chamber at the start of the intestinal digestion period; (*B*) amount of Fe measured in the bottom chamber of "companion plates" (i.e., no cells present) 2 h after the start of the intestinal digestion period; (*C*) Caco-2 cell ferritin formation as measured 24 h after the start of the intestinal digestion period; (*D*) plot of cell Fe content vs. cell ferritin as measured 24 h after the start of the intestinal digestion period. Pearson $r = 0.93$.

uptake. Conversely, cells cultured in high Fe media exhibited high ferritin content and a lower rate of iron uptake. These studies clearly demonstrated that Caco-2 cells synthesize ferritin in response to iron uptake in proportion to the Fe content of the culture medium.

The culture conditions used in this study were designed to generate cells of relatively low Fe status. The culture media contained low amounts of available Fe; therefore the cells formed minimal amounts of ferritin and exhibited near maximal rates of Fe uptake. Iron uptake and ferritin content of cells cultured under these conditions have been characterized previously (Gangloff et al. 1996). It was necessary to establish these conditions if we were to adequately test our hypothesis that ferritin formation can serve as a measure of Fe uptake and availability. These conditions allow for maximal sensitivity of our system to detect differences in food Fe availability.

Ascorbic acid has long been known to be a strong enhancer of iron uptake and availability; thus, it was used in this study to test for a positive relationship between ferritin formation and Fe uptake. The results of Figure 2 clearly demonstrate an increase in ferritin formation associated with the amount of Fe placed in the upper chamber (Fig. 2*A*) or that which diffused into the bottom chamber (Fig. 2*B*). The results shown in Figures 2C and 2*D* indicate that much of the Fe that diffused into the bottom chamber was taken up by the cell monolayer. Because ferritin formation is positively associated with cell Fe content (Figs. 2*D*, *E* and *F*), the results demonstrate that ferritin formation is representative of cell Fe uptake.

The results of Figure 2 also indicate that maximal Fe uptake from these conditions may occur with between 50 and 100 μmol/L Fe in the digest. For example, in the presence of cells, the digest containing 100 μmol/L Fe was the only digest that exhibited values greater than the "blank" control digest for the amount of bottom chamber Fe (Fig. 2C). The amount of ferritin formation supports the above conclusion because ferritin values were not different between the 50 and 100 μmol/L digests (Fig. 2*E*). Additional experiments would have to be conducted to confirm this observation. Such an observation could be important for foods with high Fe content and availability because it defines the maximal amount of food that should be present in the digest under these conditions. Exceeding this amount may result in less accurate measurement of Fe availability.

To further test the relationship between ferritin formation and Caco-2 cell Fe uptake, $FeSO_4$ in the presence of citric acid was added at varying concentrations to the digests. Like most ferric iron chelates, citric acid has been shown to keep Fe soluble yet in a less available form than Fe in the presence of ascorbic acid (Alvarez-Hernandez et al. 1991). Thus, it was expected that Fe uptake from these digests and the subsequent ferritin formation would be less than that of the Fe-ascorbate digests. As is evident in Figures 2 and 3, Fe in solution (i.e., Fe in bottom chamber, no cells present) was similar for both forms of Fe at equal digest Fe concentrations, yet ferritin formation was greater in cells exposed to the Fe-ascorbate digests. These results demonstrate expected differences in ferritin formation, based on the known relative Fe availability of the above digests.

One possible advantage of measurement of ferritin formation vs. quantification of radiolabeled Fe uptake is the issue of nonspecific binding of the radiolabeled Fe to the cell surface. In all Fe uptake studies, it is possible that some Fe from the uptake solution simply binds to the cell surface and is not truly taken up by the cell. Although methods exist to remove nonspecifically bound Fe from the Caco-2 cell surface, no studies have been conducted to determine if significant amounts of surface-bound iron remain after such treatments (Glahn et al. 1995). Measurement of ferritin formation circumvents the

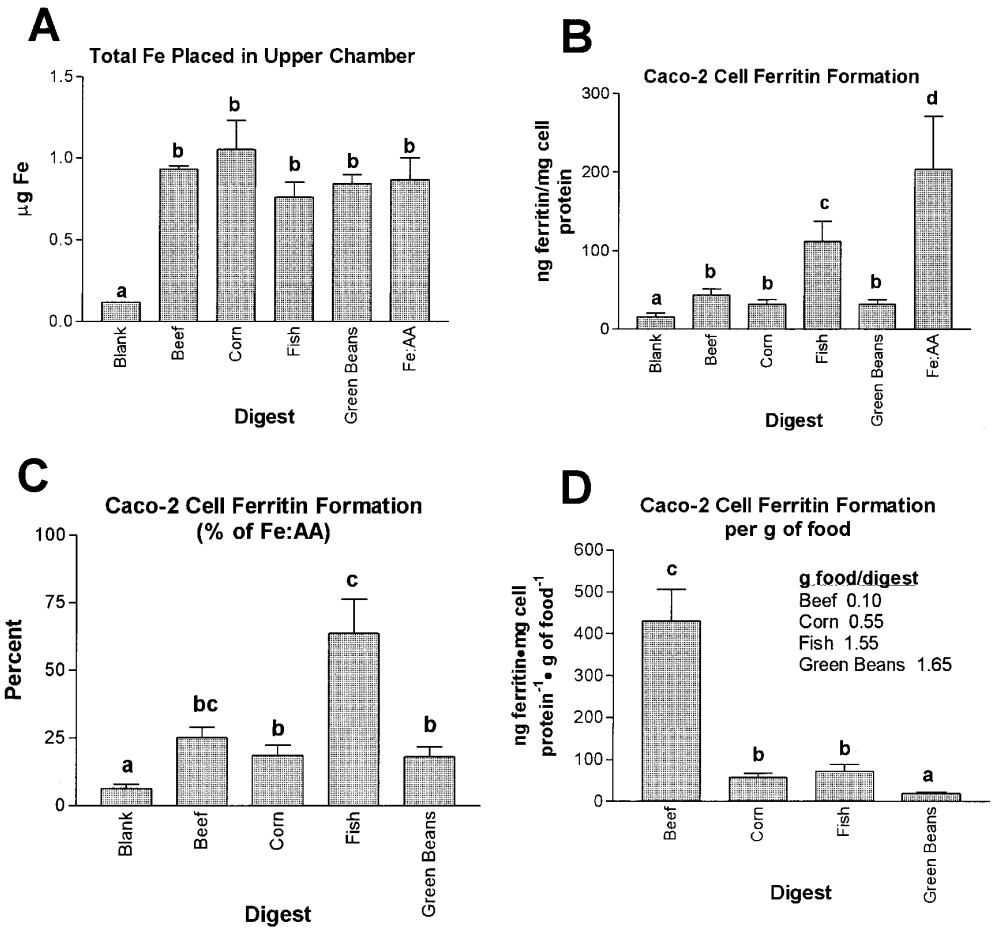

FIGURE 4 Measured variables for experiments comparing digests of beef, corn, fish and green beans. Digests were designed to include 10 μg Fe from each food. A digest containing 10 μg of Fe added as $FeSO_4$ and 1 mmol/L ascorbic acid (Fe:AA) was used as a positive control and reference standard. "Blank" indicates digest system components only (i.e., pepsin, pancreatin, bile extract), no added Fe or food. Bars (mean ± SEM, $n = 5$) with no letters in common are significantly different ($P < 0.05$). Specific panels are defined as follows: (*A*) amount of Fe measured in 1.5 mL of digest placed in the upper chamber; (*B*) Caco-2 cell ferritin formation as measured 24 h after the start of the intestinal digestion period; (*C*) Caco-2 cell ferritin formation as measured 24 h after the start of the intestinal digestion period and expressed as a percentage of the Fe:AA control; (*D*) Caco-2 cell ferritin formation as measured 24 h after the start of the intestinal digestion period and expressed per gram of food used in each digest.

issue of nonspecific binding because it is known to be a response to iron already taken up by the cell (Beard et al. 1996).

The results of Figure 4 further demonstrate the usefulness of ferritin formation as a measure of Fe availability. In these studies it was expected that higher Fe availability would be evident from digests of beef and fish relative to corn and green beans if ferritin formation is to be a useful method for measuring Fe availability. To properly interpret these data, it is important to remember that these foods differed greatly in Fe content. Thus, different amounts of food were added to the digest to attain identical digest Fe concentrations (Fig. 4*A*). As shown in Figures 4*B* and *C*, the fish digest caused the greatest amount of ferritin formation relative to the beef, corn and green beans; however, on a per gram of food basis, the beef digest clearly induced the greatest amount of ferritin formation (Fig. 4*D*). In addition, the fish and green bean samples were very similar in Fe concentration, and the fish digest induced more ferritin formation than the green bean digest. From a nutritional standpoint, these results demonstrate greater Fe availability from animal tissue and thus support the use of ferritin formation as a measure of Fe uptake in this system.

We discovered that to utilize ferritin formation as a method for measuring nonradiolabeled Fe uptake, the Fe content of the digestive enzymes had to be drastically reduced. For example, pilot studies had determined that the pepsin purchased from Sigma contained 54 μg Fe/g of pepsin. If untreated, a pepsin solution as used in our model would contribute enough Fe to the digest such that ferritin formation from a blank (no food or added Fe) digest containing ascorbic acid at 1 mmol/L would be four- to fivefold greater than that from a blank digest without ascorbic acid. Such an effect could significantly alter the accuracy and interpretation of data in this model system and thus negate the usefulness of this model for foods of low Fe concentration. As a result, both the pepsin and pancreatin-bile solutions were treated to remove Fe (see Materials and Methods). These methods were successful in removing >95% of the Fe from the pepsin solution, and >45% of the Fe from the pancreatin-bile solution. In spite of treatment of these solutions, a significant increase in ferritin formation was observed from cells exposed to a blank digest with 1 mmol/L ascorbic acid vs. cells exposed to a blank digest without ascorbic acid (Fig. 2*E*). However, the digest containing 10 μmol/L Fe with ascorbic acid exhibited approximately three times the amount of ferritin vs. the digest containing only ascorbic acid. The above results demonstrate the sensitivity of this method and emphasize the importance of maintaining minimal Fe contamination in this model system. Digest conditions that contain ample amounts of an iron uptake promoter can significantly increase the baseline measurement simply by acting on the contamination iron present in the system. For the purposes of this study, the pepsin and pancreatin-bile solutions were deemed sufficiently free of Fe for this model system.

Because Zn may interfere with Fe uptake (Wien et al. 1994), the Zn concentration of the pepsin and pancreatin-bile solutions were also measured and found to be similar to values observed for Fe. Removal of Zn from the pepsin and pancreatin-bile mixture was 99 and 64%, respectively. The authors strongly recommend that commercial preparations of these enzymes be cleaned of Fe and Zn before use in this system. In our experiments, we routinely measured Fe concentration in the enzyme solutions and found 0.07 mg Fe/L to be an acceptable level for both the pepsin and pancreatin-bile preparations.

Although detailed studies of Zn uptake using this model system have not been done, it is reasonable to assume that contamination levels similar to those for Fe should be acceptable.

Also of importance with reference to contaminant Fe present in the system was the concentration of Fe in the cell culture media (MEM) present on the cells during the intestinal digestion period. Pilot studies showed that it was important to use MEM with no Fe in its formulation (see Materials and Methods). An acceptable level of Fe in the MEM was found to be ≤ 8 μg/L.

Measurement of ferritin formation by Caco-2 cells is very sensitive. For example, measurement of ferritin formation from each culture well required only 10 μL from a total sample size of 2 mL. If needed, the ferritin assay could easily accommodate a sample size of 50 μL and possibly up to 100 μL. In addition, it is important to note that for the foods studied (Fig. 4), only 10 μg Fe was present in the digests and only 1 μg of that Fe was placed in the upper chamber of each culture well. Thus, the specific conditions such as digest tube size, Caco-2 cell monolayer size, incubation time and volume used in the upper chamber appear adequate and able to accommodate foods with relatively low Fe concentrations. This is an important feature because too much food may result in a pasty digest from which soluble Fe may not easily diffuse. It is also important to note that the foods studied here were very different (plant vs. animal tissue) and covered a broad range of Fe concentrations, thus demonstrating the versatility of this method. Although not particularly difficult, we have found comparisons of plant and animal tissue to be more complex because of the texture and overall difference between the foods. Particular care should be taken to achieve a representative sample when pipetting the digest into the upper chamber. This system appears especially well-suited for comparisons of similar types of foods such as wheat vs. corn, or comparing several varieties of beans, pharmaceutical Fe supplements and foods such as infant formula and infant cereals (Glahn et al., unpublished observations).

In this model system, the size (area) of the Caco-2 cell monolayer and the ability of the cell monolayer to take up nutrients are major factors in defining the specific conditions of the in vitro digestion. For example, in this study, the cell monolayer of each well was cultured on an area of 9.4 cm^2, which resulted in $\sim$2 mg of cell protein per well. The results in Figure 2, which document one of the most available forms of Fe, indicate that maximal rate of Fe uptake occurred between 50 and 100 μmol/L Fe in the digest. Thus, until additional experiments can be conducted to characterize uptake values at or above this concentration range, it may be wise to keep the Fe concentration of the digest below 50 μmol/L to ensure maximal accuracy of this system. Fortunately, an Fe concentration of 50 μmol/L in a 15 mL digest results in 41.9 μg Fe, which is sufficient to enable study of foods with high levels of Fe. Overall, the results demonstrate that the specific conditions used in this study allow for estimation of Fe availability from a diverse range of foods.

In summary, Caco-2 cell ferritin formation is a highly sensitive marker of Fe uptake and enables measurement of Fe availabilty in this in vitro system. This method also eliminates the need for extrinsic or intrinsic labeling of the food to determine Fe availability. The low cost, ease of use and widespread acceptance of the Caco-2 cell line make this model system an attractive alternative to animal studies and a valuable tool for use in conjunction with human trials.

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