The Clearance of $^{131}I$-Human Plasma Ferritin in Man

By Mark Worwood, Susan J. Cragg, Ann M. Williams, Michael Wagstaff, and Allan Jacobs

Ferritin was purified 33,000-fold from the plasma of patients with idiopathic hemochromatosis. The plasma ferritin was labeled with $^{131}I$ and injected into 2 normal men. Clearance was found to be relatively slow, with 50% $^{131}I$-ferritin remaining in the plasma at 27–30 hr. The fraction of plasma ferritin that bound to concanavalin-A was found to be cleared more slowly than the nonbinding fraction. These findings confirm our previous suggestion that glycosylation is a major factor prolonging the survival of ferritin in the plasma, but differ from the results of earlier studies in experimental animals and preterm infants, which indicated very rapid plasma ferritin turnover.

In normal subjects, serum ferritin concentrations provide a good indication of the amount of storage iron in the body, but in some pathologic conditions, high serum ferritin concentrations are found in patients with normal iron stores (for a review see Worwood). Little is known about plasma ferritin turnover, but changes in ferritin turnover may influence the concentration of circulating ferritin. Differences between the biochemical properties of serum and tissue ferritins suggest either differential release of the various “isoferritins” from cells or differential clearance from the plasma.

Ferritin from human tissues is composed of a protein shell containing 24 subunits which encloses an iron core of up to 4500 atoms of iron. The isoferritins of varying isoelectric points (pl) are thought to be molecules with different proportions of two types of subunit, H and L. Tissue ferritins contain little carbohydrate, and glycosylation does not appear to be a cause of microheterogeneity.

Serum ferritin has mostly been studied in patients with iron overload. The protein contains molecules with isoelectric points encompassing the range found in tissues but, whatever their pl, serum isoferritins are immunologically similar to liver or spleen ferritin and do not cross-react with antibodies to heart or HeLa cell ferritin. The iron content is also very low.

Serum ferritin seems to be glycosylated, as up to 70% of normal serum ferritin binds to concanavalin-A, whereas tissue ferritins do not bind. The microheterogeneity also seems to be caused by glycosylation, as treatment with neuraminidase reduces the range of “isoferritins” seen on isoelectric focusing. Recently it has been found that purified human serum ferritin contains a carbohydrate-containing subunit (‘G’) in addition to the L subunit found in tissue ferritin.

Previous studies on the clearance of ferritin from the plasma have been carried out in several species by injecting purified liver ferritin intravenously. In rats, dogs, and rabbits it has been found that liver ferritin is removed very rapidly from the circulation ($T_{1/2}$ 2–10 min for doses of about 1 µg). The only human study reported is that of Siimes and colleagues who studied changes in plasma ferritin concentration in premature babies undergoing exchange transfusion. They calculated plasma half-lives for ferritin that varied from 3–30 min in different babies. Such clearance rates imply considerable rates of protein and iron turnover.

Because of the biochemical properties of serum ferritin, in particular its carbohydrate content and its lack of iron, it is not possible to predict its behavior from the study of liver ferritin, nor can human plasma ferritin turnover be deduced from rat studies. For these reasons we have measured directly the rate of removal of human plasma ferritin from the plasma by injecting purified human serum ferritin labeled with $^{131}I$ into two normal male volunteers. Plasma for the purification of serum ferritin was obtained from patients with idiopathic hemochromatosis, as these patients provide the only convenient source of plasma with high concentrations of ferritin and no hepatitis antigen.

MATERIALS AND METHODS

Purification of Plasma Ferritin

Ferritin was purified from 4 liters of plasma collected from three patients with idiopathic hemochromatosis who were undergoing treatment by phlebotomy. The method of purification was that described by Cragg et al. and included heat treatment at 70°C, affinity chromatography with antibodies to human spleen ferritin, gel filtration, and ion exchange chromatography.

In order to study the behavior of “partially purified” serum ferritin in rabbits, 30 ml of serum was obtained from a patient with idiopathic hemochromatosis (serum ferritin concentration approximately 3000 µg/liter) and passed through a column of Sepharose 6B (850 x 25 mm) in two batches. Ferritin eluting from the column was detected by immunoradiometric assay, and fractions containing ferritin were combined and concentrated to about 5 ml using a
Sartorius Collodion bag (Sartorius GmbH Göttingen, West Germany).

Purified plasma ferritin was subjected to polyacrylamide gel electrophoresis, carried out at pH 7.5 in 5% gels, and subunit analysis was performed using sodium dodecyl sulphate (SDS) gradient pore polyacrylamide gel electrophoresis, both methods according to Cragg et al.1 Isoelectric focusing was performed using flat-bed gels of 5% polyacrylamide.2 The gels were either fixed and stained for protein or incubated with sheep anti-human spleen ferritin before being washed and stained as described by Worwood et al.3 Binding to concanavalin-A by the purified plasma ferritin was studied by the method of Worwood et al.12 Protein was determined by the method of Lowry et al.21 using bovine serum albumin as standard, and ferritin concentration was measured by immunoradiometric assay with spleen ferritin standards and anti-spleen ferritin antibodies.23 Antibodies to human ferritin for use in immunoprecipitation were raised in sheep using methods described for rabbits by Worwood,22 and specificity was tested by immunoelectrophoresis against spleen ferritin and human serum. Iron was estimated by flameless atomic absorption spectrometry (Varian Models 1100 and 63).

Labeling of Plasma Ferritin With Radioiodine

Human plasma ferritin (approximately 50 µg protein in 30 µl of 0.05 M Tris-HCl buffer, pH 6.5, containing 0.004% chlorobenzidine as preservative) was iodinated with either Na[125]I (IMS 30; 100 mCi/ml; Amersham International Ltd., Amersham, U.K.) or Na[131]I (IBS 30; 40 mCi/ml; Amersham International Ltd.) using the chloramine-T method.22 Solutions of reagents, apart from ferritin, were prepared from sterile, pyrogen-free water and 250 µl of the bag of eluting solution above the column outlet. All connections between reservoir and column were made with sterile plastic filters (Millipore Corporation, Bedford, Mass.) before use.

After iodination, the reaction mixture, diluted with buffer containing human serum albumin (see below) and potassium iodide, was transferred to a clean class II microbiologic safety cabinet and washed with sterile, pyrogen-free water and passed through 0.22-µm filters (Millipore Corporation, Bedford, Mass.) before use.

The immunoreactivity and nonspecific precipitability of the iodinated ferritin was followed by measuring plasma radioactivity in samples taken from the other ear 2-300 min after injection and by precipitating labeled ferritin with sheep anti-human spleen ferritin. Ferritin concentrations in the rabbit plasma samples were also measured by immunoradiometric assay. It was necessary to allow radioactivity to decay before assaying for ferritin.

Clearance of Human Plasma Ferritin From the Plasma in Normal Subjects

Two normal, adult, male volunteers were each injected with [131]I-labeled human plasma ferritin (5 µCi [131]I and 1.4 µg ferritin in 1 ml of the sterile 0.9% NaCl solution containing human serum albumin), and 10-ml samples of blood were collected into heparin at 2, 4, 8, 15, 30, 60, 120, and 180 min after injection. Further blood samples were taken at intervals during the following 9 days. Each subject took potassium iodide (60 mg/day, by mouth) on the day before injection of [131]I and for the next 15 days in order to prevent uptake of radioiodine by the thyroid. Plasma was separated and duplicate samples of 1 ml were counted for radioactivity. Labeled ferritin was precipitated from duplicate samples of 500 µl plasma by adding 250 µl of sheep anti-spleen ferritin serum (diluted for optimal precipitation of carrier ferritin), and 250 µl of human spleen ferritin (100 µg/ml in 0.9% NaCl) before incubating overnight at 4°C. The precipitates formed were sedimented by centrifugation at 2,500 rpm for 15 min and washed once with 0.9% NaCl solution before counting for [131]I activity.

Measurements of binding to concanavalin-A, both in the original [131]I-labeled plasma ferritin and in the plasma samples collected during the first 2 days, were carried out by the method of Worwood et al.12 with certain modifications. Suspensions of Con-A-Sepharose (Pharmacia, Ltd.) and Sepharose 4B were prepared in 0.05 M barbital-HCl buffer, pH 8, containing NaCl (0.1 M), sodium azide (0.2 g/liter), and bovine serum albumin (5 g/liter). Plasma (0.15 ml) was incubated for 1 hr at room temperature with either Con-A-Sepharose or Sepharose 4B (50% suspension, 2.25 ml) and serum (diluted 50x in 0.9% NaCl solution), and 250 µl carrier human spleen ferritin (100 µg/ml in 0.9% NaCl) made up to 1.0 ml with 0.9% NaCl. After centrifugation and washing of the precipitate in 0.9% NaCl solution, radioactivity in the precipitate was counted and compared with the total radioactivity added to the tube.

Determination of the Distribution of Radioactivity Among Isoferritins

A small amount of plasma ferritin labeled with [131]I was added to unlabeled plasma ferritin and subjected to isoelectric focusing. After staining for protein, the gel was prepared for fluorography, which was carried out as described by Bonner and Laskey.23

Tests for Sterility, the Presence of Pyrogens, and Toxicity

Microbiologic and viral sterility was demonstrated by standard techniques. Pyrogen testing was carried out in three rabbits by SafePharm Ltd., Derby, England, as described in the British Pharmacopoeia.24 Three rabbits were injected intravenously with twice the dose to be injected in the volunteers. No signs of toxicity were observed during the following 7 days.

Injection of Plasma Ferritin Into Rabbits

New Zealand white rabbits (weighing about 3 kg) were injected, via the marginal ear vein, with 1-18 µg plasma ferritin labeled with [131]I or with partially purified, unlabeled plasma ferritin. Clearance of the iodinated ferritin was followed by measuring plasma radioactivity in samples taken from the other ear 2-300 min after injection and by precipitating labeled ferritin with sheep anti-human spleen ferritin in the presence of carrier spleen ferritin. Ferritin concentrations in the rabbit plasma samples were also measured by immunoradiometric assay. It was necessary to allow radioactivity to decay before assaying for ferritin.

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0.75 ml buffer. In some cases the volumes were doubled. After centrifugation, the supernatants were removed. Ferritin labeled with $^{131}$I was precipitated from these supernatants by addition of anti-spleen ferritin and carrier ferritin. The precipitates were washed and counted. The percentage of $^{131}$I-plasma ferritin binding to concanavalin-A was calculated from the activities present in solution after incubation with Con-A-Sepharose 4B and the activity present after incubation with Sepharose 4B alone.

The plasma volume was calculated from the radioactivity in the plasma at zero time. This was obtained by extrapolation of radioactivity of samples taken from 5 to 30 min. The methods were as described by the ICSH\textsuperscript{2} except that $^{131}$I-ferritin was injected so that the plasma volumes were determined as part of the clearance study. Analysis of the clearance curves was carried out by fitting 2 or 3 single exponential clearances using a weighted, nonlinear, least squares program.\textsuperscript{26}

The proposal for the study of $^{131}$I-labeled ferritin clearance in normal subjects was approved by the Division of Medicine, South Glamorgan Health Authority (Teaching) and the Department of Health and Social Security Isotope Advisory Panel. Both subjects were members of the research team.

### RESULTS

**Properties of Plasma Ferritin**

Comparison of polyacrylamide and isolectric focusing gels stained directly for protein and gels stained for protein after immunoprecipitation with anti-spleen ferritin antibodies confirmed that the plasma ferritin preparation was pure. The specific concentration of the plasma ferritin was 0.85 $\mu$g ferritin/$\mu$g protein. This value is dependent on the immunoreactivity of the particular ferritin standard in the assay compared to that of the sample, but is compatible with purity.\textsuperscript{3} Subunit analysis of the plasma ferritin preparation indicated that 15% of the subunits were G subunits, the other 85% being L subunits with only a trace of H subunit present. Binding to concanavalin-A was 44% and the iron content was low (0.01 $\mu$g iron/$\mu$g protein).

After radioactive labeling of plasma ferritin with $^{131}$I, 94% of the radioactivity could be precipitated with anti-spleen ferritin. Less than 0.5% of radioactivity was precipitated with the sheep IgG-anti-IgG complex in the test for nonspecific precipitation. Tests in rabbits for pyrogenic activity and toxicity were negative. HbsAg was not detected in the preparation. Preferential labeling of ferritin that binds to concanavalin-A occurred during the iodination as, although fluorography performed after isoelectric focusing showed that all the “isoferritin” bands were labeled, 60% of the labeled preparation bound to concanavalin-A compared with 44% of the unlabeled ferritin measured by immunoradiometric assay.

**Rabbit Studies**

The iodinated plasma ferritin was injected into rabbits and the rate of removal from the plasma was measured by precipitating ferritin from the plasma and (after decay of radioactivity) by immunoradiometric assay. For the first hour, clearance appeared to be a first-order process and T/2 was calculated by linear regression analysis. For the labeled ferritin, T/2 was 110 ± 25 min (5 rabbits), significantly slower than the value of 74 ± 19 found by immunoradiometric assay. This difference is probably explained by the preferential labeling of ferritin that binds to concanavalin-A, as it was found that non-concanavalin-A-binding ferritin was cleared from the plasma more quickly than Con-A-binding ferritin.\textsuperscript{18} When the partially purified serum ferritin was injected into 2 rabbits, the values of T/2 obtained were 58 and 62 min, not significantly different from those obtained by immunoradiometric assay for purified serum ferritin. The amount of ferritin injected was 7 $\mu$g, and 32% of the

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*NA, not available.

*Corrected for background counting rate and decay.
ferritin in the injection solution bound to concanavalin-A.

**Human Studies**

During the period of study, the plasma ferritin concentrations and the percentage of plasma ferritin binding to concanavalin-A remained relatively constant (Table 1). The plasma samples from each volunteer were assayed in one batch along with a control serum. This serum was independently diluted 17 times and an aliquot assayed alongside the plasma samples for each sample time. For subject 1, the mean ferritin concentration (±SD) was 138 ± 17 μg, with a coefficient of variation (CV) of 12%. For subject 2, the mean value was 87 ± 16 μg, with a CV of 18%. However, if the value for the 120-hr sample is deleted, being more than 3 standard deviations from the mean, the CV is only 10%. The CV for the control serum was also 10%. Thus, most of the variation in plasma ferritin concentration was methodological, and correction of the plasma radioactivity to allow for changes in the plasma ferritin pool is not justified. Throughout the study the percentage of 131I activity precipitated with antiferritin remained high (Table 1), and in analyzing the disappearance of plasma radioactivity, no correction was made for non-ferritin 131I.

The clearance of 131I-labeled plasma ferritin is shown in Fig. 1. The plasma volumes measured from the initial radioactivities of labeled ferritin were 3.58 liters for subject 1 and 3.08 liters for subject 2 (44 and 50 ml/kg)—within the normal range quoted by Dacie and Lewis.27 The plasma radioactivity had declined to half its original value after about 30 hr in both subjects. However, it is obvious that the removal of label from the plasma cannot be represented as a single first-order clearance. The clearance curve was therefore analyzed as described in Materials and Methods.

In the case of subject 2, there was a small proportion of 131I (10%) that was rapidly removed from the circulation (T½ = 30 min). After removal of this component it was found that for both subjects a reasonable fit with the experimental data was obtained with two single exponential clearances. For both subjects the first component (about 35% of the injected labeled ferritin) showed a half-time of 12 hr and the second component (65%) a half-disappearance time of 87 hr. Similar results were obtained by analyzing the clearance curves after correcting for the amount of 131I precipitated with antiferritin.

It is possible that components of the labeled ferritin are removed from the plasma at different rates. This was suggested by direct measurement of the clearance of Con-A-binding and non-Con-A-binding ferritin (Fig. 2, Table 1). The radioactivity associated with the fraction of ferritin that did not bind to concanavalin-A declined rapidly (T½ approximately 5 hr, assuming a
CLEARANCE OF HUMAN PLASMA FERRITIN

The preparation of highly purified plasma ferritin has made it possible to study its rate of clearance from the plasma directly, and we have measured this in two normal men.

Although ferritin has been obtained from patients undergoing treatment by phlebotomy, its properties are similar to those of normal serum ferritin. Both have a similar protein distribution on isoelectric focusing, both are immunologically similar to liver and spleen ferritin, and in both a high proportion of the ferritin binds to concanavalin-A. We have previously shown that affinity chromatography with antibodies to spleen ferritin did not select particular isoferritins from a patient with secondary iron overload.

The overall rate of clearance of labeled ferritin was relatively slow, with 50% of initial radioactivity remaining in the plasma after 30 hr. Such a rate of clearance is similar to that found for many of the tissue enzymes that are released into the plasma, but quite different from the previous studies of ferritin clearance in laboratory animals and to the rapid ferritin turnover found in premature babies by Siimes et al.

In order to make calculations from the plasma activity curves about the turnover of ferritin, it is necessary to satisfy a number of conditions: (1) The metabolism of the labeled protein is the same as that of the native unlabeled protein. (2) There is no reutilization of the label. (3) The system is in a metabolically steady state during the study. (4) Newly synthesized protein is distributed throughout the intravascular pool before entering the extravascular pool. We have provided evidence that conditions 1 and 3 have been satisfied and condition 2 has been shown to apply in normal subjects given potassium iodide during the course of the study. About condition 4, we have no information, as little is known about the origin of plasma ferritin, but we assume that this condition also applies.

Assuming that these conditions are satisfied, the clearance curves may be analyzed in various ways. For example the initial decline in radioactivity may be
taken to reflect the distribution of ferritin throughout the intra- and extravascular circulation, and the rate of degradation of the protein may be calculated from the slope of the latter part of the curve.

However, such an analysis is inappropriate because it must be assumed that the labeled ferritin molecules are cleared in a uniform way, and it is apparent from Fig. 2 that this is not the case. Ferritin, which does not bind to concanavalin-A, leaves the plasma rapidly (T/2 $\approx$ 5 hr), while the concanavalin-A-binding fraction shows a much slower rate of removal. Unfortunately, the data relating to con-A binding of ferritin do not permit us to make a reliable analysis of the separate clearance curves. Counting rates were low compared with those for the analysis of simple plasma radioactivities, and the relatively complex analysis increased the discrepancies between duplicate aliquots of plasma. Although we have not attempted further analysis of the plasma activity curves, it is likely that these reflect both exchange between intra- and extravascular circulations and catabolism of labeled ferritin with very different rates for Con-A and non-Con-A-binding ferritin molecules.

The differences between our human study and previous findings in animals are probably due to both species differences and the type of ferritin injected. In most animal experiments, liver ferritin of high iron content was injected and disappeared from the circulation within minutes. Halliday et al.\textsuperscript{16} found prolonged survival of rat serum ferritin compared with kidney ferritin, but the half-time was still less than 20 min. The very different rates of clearance calculated by Siimes et al.\textsuperscript{18} and those obtained by us are not easy to explain. During the first few weeks of life there are changes in the distribution of iron and in serum ferritin concentration,\textsuperscript{2} and these changes may also be associated with changes in the biochemical and immunologic properties of serum ferritin. Another possibility is that the labeled ferritin injected in the present study had been modified during purification. However, such changes usually cause insolubility and rapid removal from the plasma, and in rabbits, the purified serum ferritin was cleared in a similar way to the "unpurified" material.

The very different rates of clearance obtained in experimental animals and in the present study suggest that differential clearance of ferritin molecules may be the major factor determining the biochemical properties of plasma ferritin. The tissues contain large amounts of ferritin with varying iron contents and varying ratios of H:L subunits. Liver and spleen contain ferritin composed mostly of L subunits.\textsuperscript{4} Serum ferritin is immunologically very similar to liver or spleen ferritin but contains little iron and includes carbohydrate-containing molecules.\textsuperscript{3} These properties may be entirely due to the selective release of isoferritins, but the following scheme seems more likely.

1. Release of cytosolic, noncarbohydrate-containing ferritin molecules from damaged cell membranes. This may be followed by differential clearance leading to the accumulation in the plasma of only the iron-poor isoferritins (homopolymers of L subunits) of relatively high pi (the so-called "natural apoferritin"\textsuperscript{10}).
2. Carbohydrate-containing apoferritin molecules (composed of L and G subunits) may be secreted from cells after synthesis on rough endoplasmic reticulum and glycosylation.\textsuperscript{3} Whatever the origin of this fraction of plasma ferritin, these molecules have a prolonged survival in the plasma.

Thus, the ferritin that accumulates in the plasma has little iron, is composed of L and G, but with few H subunits, and shows microheterogeneity caused by variation in sialic acid composition. We have now shown that glycosylation is not only an important factor explaining the microheterogeneity of plasma ferritin but also determines the survival of ferritin molecules in the plasma.

ACKNOWLEDGMENT

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