Serum transferrin receptor (TfR) is mostly derived from bone marrow erythroblasts, and TfR concentrations are increased by both enhanced erythropoiesis and iron deficiency (1–5). Several studies have shown that serum TfR measurements are especially useful in the differential diagnosis of iron deficiency anemia (IDA) and anemia of chronic disease (ACD) (6–8). Soluble TfR released by cleavage of membrane receptors between amino acids 100 and 101 (Arg-Lys) just above the cell membrane and purified from human serum is able to rebind with transferrin (Tf) (9–11). However, details regarding the molecular structure of the soluble TfR present in serum or plasma have not been clarified. Previously it was shown that in serum, truncated TfR exists as a complex with Tf (9, 11), and we have demonstrated by HPLC size fractionation that the predominant form of serum TfR is a dimeric TfR in complex with Tf (12). A study on the binding of radiolabeled Tf to the surface TfR of K562 human myelogenous leukemia cells showed that the binding affinity of monoferric Tf was reduced to approximately one-fourth that of diferric Tf and that the binding affinity of apo-Tf was nearly zero (13, 14). Therefore, it is plausible that in serum, Tf-TfR binding affinity is decreased under conditions associated with decreased Tf iron saturation and that this may affect the ratio of these two substances in complex formation. In the present study, we examined whether there is a difference in the molecular structure of serum TfR between healthy individuals and patients with decreased Tf iron saturation, such as in cases of IDA and ACD.

Blood samples were collected from patients who were diagnosed as having IDA (n = 40; defined as serum ferritin <12 μg/L and C-reactive protein <7 mg/L) or ACD [n = 16; serum ferritin (mean ± SD), 80 ± 66 μg/L; C-reactive protein, 34 ± 30 mg/L] caused by collagen diseases, including rheumatoid arthritis. The study protocol had been approved by the local ethics committee. Serum was separated from blood and kept frozen at −20 °C until used. Complete blood counts, serum iron, and serum ferritin were determined by routine methods.

The serum TfR concentration was determined by an ELISA based on a method described previously (12). In this assay, protein calibrators (TfR-Tf complex) were purified from pooled human plasma in which Tf was saturated with excess iron. Anti-TfR monoclonal antibody (clone 16E; 10 μg/mL) was coated on a microtiter plate. Samples or calibrators were diluted 100-fold with phosphate-buffered saline containing 10 g/L bovine serum albumin, and a 100-μL aliquot was pipetted into each well and incubated for 2 h at 37 °C. The plate was washed three times with phosphate-buffered saline containing 1 mL/L Tween-20, and 100 μL of peroxidase-labeled rabbit anti-TfR polyclonal antibodies (0.1 mg/L in phosphate-buffered saline) was added. The mixture was then incubated for 2 h at 37 °C. The plate was washed again, and color was developed with 0.42 mmol/L tetramethylbenzidine and 1 mmol/L H2O2 in 0.1 mol/L citrate buffer, pH 6.0) for 30 min at room temperature. After the reaction was stopped by the addition of 50 μL of 2 mol/L H2SO4, the absorbance was measured at 450 nm. The serum TfR values in samples were calculated based on the ratio of TfR to the TfR-Tf complex.

We generated the reference interval (median 95%) for the newly developed TfR assay by analyzing the serum TfR values in 175 healthy adults. The reference intervals were 0.6–1.2 mg/L for serum TfR and 0.3–0.8 for the TfR-ferritin index (TfR/[log] ferritin), which has been suggested as a highly sensitive index of iron status (8), and the results correlated well with the Amgen R&D Systems ELISA assay (r² = 0.964). The mean (± SD) TfR concentrations in patients with IDA (3.16 ± 1.49 mg/L) and ACD (1.25 ± 0.48 mg/L) were significantly higher than those in the healthy adults mentioned above (0.84 ± 0.21 mg/L). The IDA and ACD groups were well distinguished from each other in terms of ferritin (area under the ROC curve, 0.995; Stat Flex for Windows software package), serum TfR (area under the ROC curve, 0.92), or TfR-ferritin index (area under the ROC curve, 0.998). When we used a combination of serum iron and TfR-ferritin index, IDA and ACD patients and healthy individuals were remarkably distinguishable from each other (data not shown).

Because of the decrease in serum iron, mean (± SD) iron saturation of Tf decreased to 7% ± 8% and 11% ± 6% in the patients with IDA and ACD, respectively. Under these conditions, a considerable proportion of Tf may exist as monoferric Tf and apo-Tf. Because a decrease in iron saturation of Tf decreases the binding affinity of Tf to TfR, we examined the molecular form of the circulating Tf-TfR complex in relation to iron status. Serum samples were size-fractioned by HPLC as described previously (12), and the TfR-Tf complex that eluted from the column was detected by the ELISA for TfR. In sera from healthy individuals, TfR-Tf eluted as a single peak at ~330 kDa (Fig. 1A); this indicates that in serum, two molecules of TfR were bound to two molecules of Tf, taking into consideration that the molecular mass of one TfR molecule is 85 kDa and that of Tf is 80 kDa. However, how the dimerized serum TfR is actually formed is not understood...
because there is no cysteine residue that can form a disulfide bridge on the C-terminal side of the truncated TfR (10). Interestingly, when serum from a patient with severe IDA was analyzed in a comparable manner, the TfR-Tf complex eluted in a rather broad range at ~250 kDa, suggesting the predominance of a 2:1 TfR-Tf complex. We incubated IDA serum with 40 mg/L ferric citrate before immunoprecipitation to determine whether an increase in iron concentration changes the composition of the Tf-TfR complex. After preincubation with ferric citrate in an amount sufficient to saturate serum Tf with iron, the same IDA sample showed a shift of the TfR peak to ~330 kDa. This suggests that iron saturation of Tf restored the ratio of TfR to Tf in the complex to 2:2.

To further confirm the molecular form of the complex, we performed immunoprecipitation and Western blotting experiments with two anti-TfR antibodies that recognize TfR as well as the TfR-Tf complex (12). The TfR-Tf complex was immunoprecipitated from serum (25 µL) by the addition of 10 µL of polyclonal anti-TfR antibodies (12) immobilized on Sepharose beads (produced by binding 10 g/L anti-TfR polyclonal antibodies with NHS-Sepharose; Pharmacia). After immunoprecipitation, the samples were electrophoresed on 6% polyacrylamide gels containing 10 g/L sodium dodecyl sulfate and 50 mL/L 2-mercaptoethanol, and proteins in the gel were transferred to a nitrocellulose membrane for detection using horseradish peroxidase-labeled anti-TfR antibody (16E) or horseradish peroxidase-labeled anti-Tf antibody (12). Under reducing conditions, the TfR-Tf complex that was immunoprecipitated from serum by polyclonal anti-TfR antibodies was visualized as an 80- to 85-kDa band (TfR, 85 kDa; Tf, 80 kDa) (12).

For the cross-linking experiments, bis-sulfosuccinimidyldiisocyanate (BS3) was added as a cross-linker to the serum sample before immunoprecipitation. In healthy individuals, the sample was treated with ≥4 mmol/L BS3, a single polymeric substance (~330 kDa) was formed, and the complex could be visualized by either anti-TfR or anti-Tf antibodies (Fig. 1B). Consistent with the HPLC analysis, after BS3 treatment the major fraction of TfR immunoprecipitated from severe IDA serum migrated as a diffuse band at a site corresponding to a slightly lower molecular mass (~250 kDa), indicating the presence of TfR-Tf complex with a 2:1 molecular ratio. In addition, there was another diffuse band in the region of lower molecular mass (160–170 kDa), suggesting that dimeric TfR devoid of Tf may also be present. Again, addition of ferric citrate to the IDA serum before immunoprecipitation restored the size of the complex to ~330 kDa. Furthermore, in several serum samples from IDA patients, incubation with ferric citrate increased the Tf content of the TfR-Tf complex by 20–60% on the basis of densitometric image analysis. Taken together, these results suggest that in healthy individuals, serum TfR is in complex with Tf with a molar ratio of 2:2 (~250 kDa). Decreased iron saturation of Tf is associated with the appearance of TfR-Tf complexes with 2:1 molar ratios (250 kDa), which is the predominant form in IDA.

Serum TfR measurements are useful in the evaluation of iron status in cases where there is an iron deficiency or an iron overload. To date, a major problem for TfR assays has been the lack of international standardization. The present study demonstrates that iron status influences the molecular structure of the soluble TfR-Tf complex in the circulation; this information is crucial for the standardization of TfR assay systems. When we determined TfR concentrations in 22 fresh IDA serum samples that had been saturated with excess iron, the pretreatment (3.29 ± 1.61 mg/L) and posttreatment (3.26 ± 1.5 mg/L) values were the same. However, it has been shown that the immunoreactivity of serum Tf decreases significantly unless TfR is complexed with excess Tf after purification.
Plasma Homocysteine Concentrations in Patients with Liver Cirrhosis, Natàlia Ferré,1 Frederic Gómez,2 Jordi Camps,6 Josep M. Simó,1 Michelle M. Murphy,7 Joan Fernández-Ballart,2 and Jorge Joven1 (1Centre de Recerca Biomèdica and 2Unitat de Medicina Preventiva, Facultat de Medicina i Ciències de la Salut, Institut de Recerca en Ciències de la Salut, Hospital Universitari de Sant Joan, C/. Sant Joan s/n, 43201- Reus, Catalunya, Spain; 6 author for correspondence: fax 34-977-312569, e-mail jcamp@grupsgs.com)

Plasma homocysteine (tHcy) is a marker of folate or cobalamin deficiency states (1) and a risk factor for cardiovascular diseases (2), and is altered by renal insufficiency (3). Increased tHcy in liver diseases may also play a role in hepatic disorders (4, 5). Chronic treatment of experimental animals with ethanol or CCl4 is associated with hyperhomocysteinemia, and the hepatoprotective effect of S-adenosylmethionine on experimental cirrhosis is accompanied by a normalization of methionine metabolism and a decrease in tHcy concentration (4, 5). Studies in cultured hepatocytes suggest a role of the liver in metabolism of Hcy (6). However, the presence and degree of hyperhomocysteinemia in patients with liver disease and its modulation by chronic alcohol intake are, as yet, not well defined.

We studied 76 patients with liver cirrhosis (55 men and 21 women; age range, 57 ± 11 years) who were being treated in the outpatient clinic of Hospital Universitari de Sant Joan. The diagnosis of cirrhosis was based on liver biopsy or on clinical evidence, including echography to evaluate splenomegaly and portal vein dilation and fiberoptic gastroscopy to detect the presence of gas troesophageal varices. The etiology of cirrhosis was alcoholic in 48 patients (63%) and nonalcoholic in 28 (37%). Twenty-nine of the 48 alcoholic patients studied had stopped alcohol consumption at least 3 months before the study; the other 19 had continued drinking. The severity of the liver disease was measured in all patients by the Child–Pugh score (7). This classification estimates the severity of cirrhosis based on biochemical and clinical indices. The concept is to give a numeric score to certain aspects of liver function (plasma albumin and bilirubin concentrations, prothrombin time, and the degree of ascites and hepatic encephalopathy). Adding these numbers together provides a final score of liver cell function (7). The control group consisted of 83 healthy volunteers (36 men and 47 women; age range, 42 ± 14 years) participating in an epidemiologic study being conducted in our area.

In all participants, venous blood was collected, after an overnight fast, into sodium EDTA-containing tubes kept at 4°C for tHcy, folate, and cobalamin determinations, or into tubes with anticoagulants added for the other biochemical tests. The tubes were centrifuged at 2500g at 4°C, and plasma or serum was stored at −80°C. This handling minimizes preanalytical errors in tHcy measurements (8).

tHcy was measured by fluorescence polarization immunoassay (9). Folate and cobalamin concentrations were measured by ion-capture and microparticle immunoassay, respectively, in an AxSYM® analyzer (Abbott Laboratories). Serum alanine aminotransferase, alkaline phosphatase, and γ-glutamyltransferase activities and albumin and bilirubin concentrations were measured by standard techniques (ITC Diagnostics). Hemoglobin and erythrocyte mean corpuscular volume were measured in a Coulter® STKS counter (Beckman Coulter).

Means were compared by a linear multivariate model taking into account age and gender. Pearson correlation coefficients were used to evaluate the degree of association between pairs of variables. When the distribution of variables was skewed, statistical analysis was performed using log-transformed data. All calculations were performed with the SPSS 10.0 statistical package.

As expected, mean serum bilirubin concentrations and mean alanine aminotransferase activities were higher in cirrhotic patients than in the controls (Table 1). The mean corpuscular volume of red blood cells and mean serum γ-glutamyltransferase activity were significantly increased, especially in nonabstaining alcoholic cirrhotics.