

Immunohistochemical evidence of lactoferrin in hepatic biopsies of patients with viral or cryptogenetic chronic liver disease

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Summary. Lactoferrin (Lf) expression has been immunohistochemically investigated in 117 formalin-fixed paraffin-embedded liver biopsies obtained from an equal number of patients affected by chronic hepatitis (HCV=76; HBV=17; HBV+HDV=14; cryptogenetic=10); in addition, 10 autopsic specimens of normal liver were studied as control. The Lf immunoreactivity was evaluated by an intensity-distribution (ID) score. The Lf immunoreactivity was observed in 88 out of 117 (75%) cases of chronic hepatitis; interestingly, all liver specimens from HBV hepatitis showed a constant Lf reactivity with the highest ID-score, whereas the evidence of Lf was encountered in 54/76 (71.1%) HCV as well as in 11/14 (78.6%) HDV chronic hepatitis, thus documenting a variable degree of Lf immunostaining in relation to different viruses. Moreover, in 6/10 (60%) cases of cryptogenetic hepatitis Lf immunoreactivity was documented, whereas all normal liver controls were unreactive. In HCV specimens, the Lf nuclear immunoreactivity appeared to increase with the progression of the disease, with a greater expression in genotype 1. In contrast, no relationship among Lf ID-scores and different stages or grades of HBV, HDV or cryptogenetic hepatitis was encountered. This fact may suggest a role for Lf as an unspecific defensive agent in chronic inflammatory liver diseases, similarly to that elsewhere reported in other inflammatory tissue injuries.

Key words: Lactoferrin, Chronic liver disease, Liver biopsy, Immunohistochemistry

Introduction

Lactoferrin (Lf) is a major iron-binding glycoprotein detectable by radioimmunological and immunoenzymatic procedures in many biological fluids (Masson

et al., 1966; De Vet and Van Gool, 1974; Brock, 1980). By immunohistochemistry, the distribution of Lf has been investigated in several normal adult and foetal human tissues (Mason and Taylor, 1978; Reitano et al., 1980) as well as in different neoplastic conditions (Caselitz et al., 1981; Barresi and Tuccari, 1984, 1987; Rossiello et al., 1984; Charpin et al., 1985; Tuccari and Barresi, 1985; Loughlin et al., 1987; Tuccari et al., 1989, 1992, 1997, 1999; Cabaret et al., 1992; Asato de Camargo et al., 1996).

Many different unique functions have been attributed to Lf, including DNA and RNA binding, and transport into the nucleus, where Lf binds to specific sequences and activates transcription (Penco et al., 2001). Moreover, other biological functions of Lf have been addressed including regulation of iron homeostasis, antioxidant and immunoregulatory activities, bacteriostatic and bactericidal effects as well as the capability to curb the proliferation of other microbes such as fungi and viruses (Brock, 1995; Levay and Viljoen, 1995; Lonnerdal and Lyer, 1995). In particular, Lf inhibits *in vitro* the replication of a number of viruses including herpes simplex virus (HSV), rotavirus, hepatitis C virus (HCV) and even human immunodeficiency virus (HIV) (Defer et al., 1995; Fujihara and Hayashi, 1995; Ikeda et al., 1998; Tanaka et al., 1999).

In normal liver, Lf has been immunocytochemically revealed in vascular endothelium and non-parenchyma cells (Penn et al., 1996), while it has never been detected in hepatocytes, where other iron-binding proteins such as transferrin and ferritin are easily found (Mason and Taylor, 1978). However, the immunohistochemical evidence of Lf has been reported in hepatocytes of liver biopsy specimens from patients affected by primary sclerosing cholangitis or inflammatory bowel diseases (Penn et al., 1996).

In the present study, we have investigated the immunohistochemical distribution pattern of Lf in a retrospective series of human liver biopsy specimens from patients affected by viral or cryptogenetic hepatitis.

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Materials and methods

One hundred and seven bioptic specimens of liver, obtained from an equal number of patients affected by chronic viral hepatitis, were taken from files of our institutions and included in the study. In particular, seventy-six patients (M:F 59/17; age range 22-75 yrs, mean 46.9) were affected by HCV-hepatitis, seventeen (M:F 15/2; age range 20-56 yrs, mean 42.1) by HBV-hepatitis and fourteen (M:F 14/0; age range 22-63 yrs, mean 35.6) showed a delta superinfection in HBV-hepatitis (HDV). Moreover, ten patients (M:F 6/4; age range 32-58 yrs, mean 46.6) affected by cryptogenetic chronic hepatitis, in which silent type B hepatitis has been excluded, were also tested. The histopathological grading and staging of chronic hepatitis were performed according to criteria suggested by Ishak et al. (1995); clinical and serological data of all patients were also available. None of the patients we studied had been treated with antiviral or immunosuppressive drugs before undergoing liver biopsy. Finally, 10 autoptic unaffected liver samples were utilised as normal control.

Patients who had HCV-related chronic liver disease, had no detectable HBsAg, did not drink alcohol to excess or use intravenous drugs and were not infected with the human immunodeficiency virus. HCV infection was defined by the presence of anti-HCV antibodies and by the identification of plasmatic HCV-RNA utilising PCR amplification (Amplicor-HCV-Monitor, Roche, Basel, Switzerland); thirty HCV-RNA positive cases were subsequently genotyped by restriction fragment length polymorphism (RFLP) analysis, as previously described (Thiers et al., 1997). Patients affected by HBV infection were diagnosed when circulating hepatitis B surface antigen (HBsAg) was identified. Patients affected by HDV were diagnosed when HDV antigen was found in liver sections by immunohistochemical methods, as elsewhere reported (Villari et al., 1989). Patients affected by cryptogenetic hepatitis were diagnosed on the basis of negativity of the above mentioned viral markers (HBV, HCV, HDV), by negativity of the non organ-specific antibodies and by having no other independent factors of liver damage such as hepatitis abuse of alcohol consumption, α_1 -antitripsin deficiency, or haemosiderosis. All patients were negative for antibodies to human immunodeficiency virus and had no evidence of hepatocellular carcinoma.

All liver specimens, fixed in 10% neutral formalin for 12-24 hours at room temperature, were embedded in paraffin at 55 °C and cut into 4-5 μ m-thick sections; haematoxylin-eosin and Perls Prussian blue Ferrocyanide methods were routinely carried out. For the immunohistochemical study, sections were treated in a moist chamber for 30 min. each time: 1) with 0.1% H₂O₂ in methanol to block the intrinsic peroxidase activity; 2) with normal sheep serum to prevent unspecific adherence of serum proteins; 3) with rabbit anti-human Lf (w.d. 1:300) (purchased from Dako,

Copenhagen, Denmark); 4) with sheep anti-rabbit immunoglobulin antiserum (Behring Institute; w.d. 1:25); and 5) with rabbit anti-horseradish PAP complexes (Dako; w.d. 1:25). For the demonstration of peroxidase activity the sections were incubated in darkness (Weir et al., 1974) for 10 min. with 3-3' diaminobenzidine tetra hydrochloride (100 mg in 200 ml 0.03% hydrogen peroxide in PBS) (Sigma Chemical Co., St. Louis, MO, USA). To test the specificity of Lf immunostaining in order to deny the possibility of non-specific reaction, serial sections of each affected liver specimen have been tested by replacing the specific antiserum by either phosphate-buffered saline, normal rabbit serum or absorbing with excess of purified human Lf from human liver and spleen (Sigma Chemical Co.) as well as with pre-absorbed primary antibody: the results obtained were negative.

Immunostained sections were estimated by light microscopy using a x20 and x40 objective lens and x10 eyepiece. Two pathologists using a double-headed microscope performed the assessment of immunostained sections on a consensus basis. The percentage of stained nuclei and cytoplasm of hepatocytes (staining score) was separately graded as follows: 0 (no staining); 1 (>0 to 5%); 2 (>5 to 50%); and 3 (>50%). Successively, an intensity-distribution (ID) score was calculated by multiplying, for each case and different cellular district, the staining score by the staining intensity (weak=1; moderate=2; strong=3), similarly to that elsewhere reported (Douglas-Jones et al., 1995; Tuccari et al., 2000). When the results obtained in the nucleus and in the cytoplasm of each sample were not equivalent, the highest value was recorded as the ID-score of hepatocytes.

The possible correlations between immunohistochemical data and clinico-morphological or biomolecular characteristics of liver specimens were investigated using non-parametric methods (chi-square test, Mann-Whitney U-test, Kruskal-Wallis H-test). A P value less than 0.05 was considered statistically significant.

Results

The Lf immunoeexpression was observed in 88 out of 117 (75 %) cases of chronic hepatitis, independently from their different aetiology or the stained cellular district (Figs. 1, 2). The Lf positivity was granular and localised in the cytoplasm in 85 cases (72.6%), while a nuclear reactive pattern was seen in 25 specimens (21.4%). Twenty-nine cases were unreactive in all considered compartments. In different types of chronic hepatitis, Lf immunostaining, expressed as ID-score values, appeared variable in hepatocytes (Table 1) with a significant P value (0.0210); a similar significant difference (P=0.0102) was appreciable at the cytoplasmic district, while no statistical difference (P=0.9192) was found in the Lf ID-score of the nucleus. However, no relationship was found between

pathological staging/grading of the whole group of chronic hepatitis and Lf ID-score.

Moreover, employing Perls' method, the iron

presence was observed in 73/117 (62.4%) cases of chronic inflamed hepatic tissue; the blue granular positivity was mainly localised in the cytoplasm of

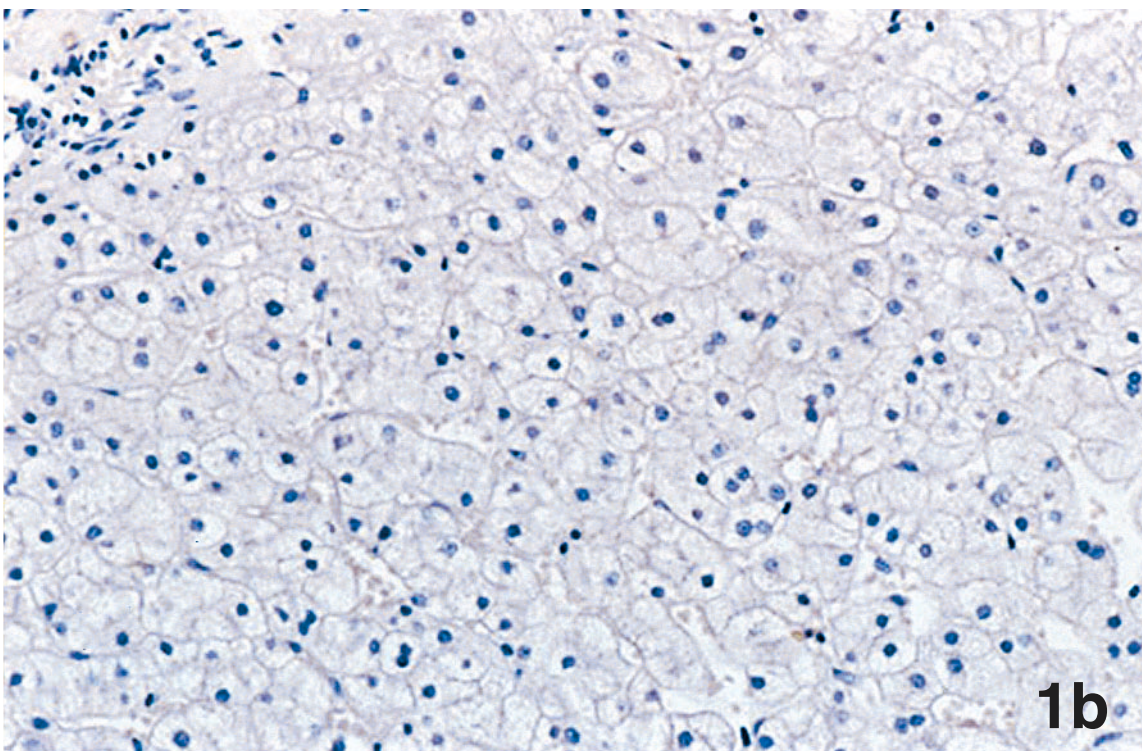
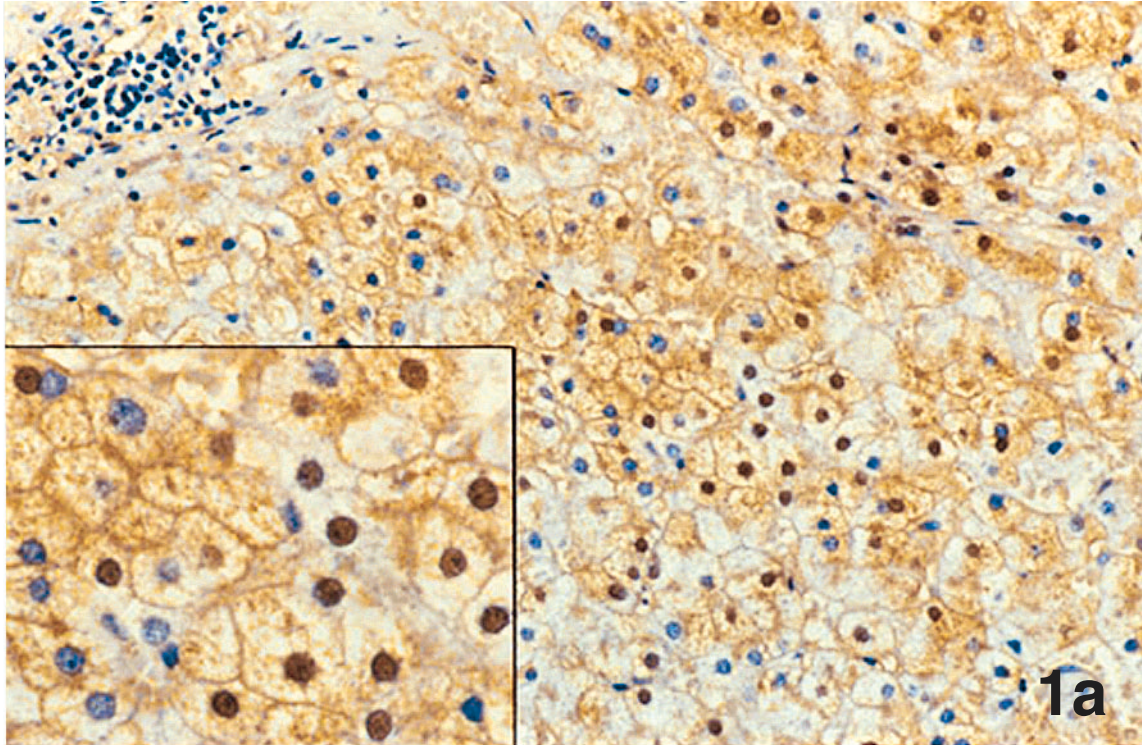


Fig. 1. Chronic HCV-hepatitis. An evident Lf immunoreactivity is encountered as granular deposits in the cytoplasm of hepatocytes (**a**), and also in the nucleus (a, inset); the corresponding control serial section with pre-absorbed primary antibody (**b**). Lf-immunoperoxidase, Mayer's haematoxylin nuclear counterstain. a, x 64; a-inset, x 128; b, x 64

hepatocytes or in perivascular tissue. No significant relationship was noted between iron deposits and Lf immunoeexpression.

In HCV-related liver specimens (Fig. 1a,b) the positive rate of Lf expression was 71.1% (54/76 cases); a direct significant relationship ($P = 0.02$) among different stages of the disease and the Lf ID-score relative to the nuclear localisation was found. After genotyping of 30 HCV cases, 16 cases were infected by genotype 1, while 14 were attributable to genotypes 2-6. In hepatocytes, Lf immunoeexpression was detected in 12/16 cases of genotype 1 and in 10/14 of other genotypes; moreover, a statistically significant difference was appreciable in the ID-score relative to the nucleus between these two groups, with a greater score in genotype 1 ($P = 0.04$).

In all HBV-related liver specimens (Fig. 2), a constant cytoplasmic localisation of Lf was observed, while 4/17 cases showed a nuclear Lf immunostaining. No relationships were found among different stages or grades of the disease and Lf ID-scores.

The Lf positive rate in hepatocytes of cases affected

by HDV or by cryptogenetic hepatitis was 78.6% (11/14) and 60% (6/10) respectively. No relationship among Lf ID-scores and different stages or grades of HDV or cryptogenetic hepatitis was encountered.

Data concerning Lf immunoeexpression in different stages of disease regarding each hepatitis group are reported in Table 2.

Finally, samples of autoptic unaffected liver were always unreactive for Lf.

Discussion

Our study represents the first immunohistochemical report concerning Lf distribution in liver of patients affected by chronic hepatitis. Lf has been detected in 75% of cases, mainly localized in the cytoplasm of hepatocytes and sometimes in the nucleus, whereas no Lf immunostaining has been encountered in normal liver autoptic specimens. The site of Lf immunoreactivity both in the nucleus and cytoplasm is not surprising in our opinion, since this secretory protein has been

Table 1. Immunohistochemical detection of Lf in normal and affected liver specimens.

	HEPATOCTE		NUCLEUS		CYTOPLASM	
	Positive rate	ID score (median)	Positive rate	ID score (median)	Positive rate	ID score (median)
Normal liver	0% (0/10)	0	0% (0/10)	0	0% (0/10)	0
HCV hepatitis	71.1% (54/76)	2	22.4% (17/76)	0	68.4% (52/76)	1.5
HBV hepatitis	100% (17/17)	4	23.5% (4/17)	0	100% (17/17)	4
HDV hepatitis	78.6% (11/14)	2	14.3% (2/14)	0	71.4% (10/14)	2
Cryptogenetic Hepatitis	60% (6/10)	1.5	20% (2/10)	0	60% (6/10)	1.5

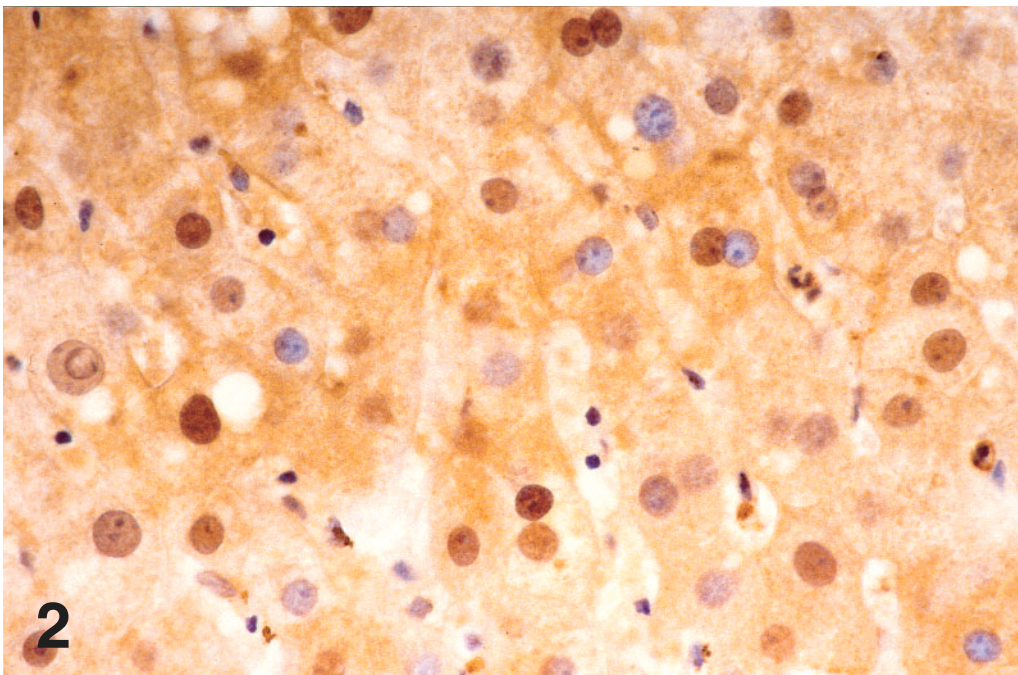


Fig. 2. Chronic HBV-hepatitis. An intense and diffuse Lf staining with a combined nuclear/cytoplasmic reactive pattern is seen in hepatocytes. Lf-immunoperoxidase, Mayer's haematoxylin nuclear counterstain. x 128

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Table 2. Immunohistochemical detection of Lf in different stages of hepatitis.

STAGE	HCV HEPATITIS			HBV HEPATITIS			HDV HEPATITIS			CRYPTOGENIC HEPATITIS		
	Positive rate			Positive rate			Positive rate			Positive rate		
	H	N	C	H	N	C	H	N	C	H	N	C
0	71% (15/21)	0% (0/21)	71% (15/21)	100% (6/6)	17% (1/6)	100% (6/6)	-	-	-	0% (0/1)	0% (0/1)	0% (0/1)
1	72% (13/18)	33% (6/18)	67% (12/18)	100% (2/2)	50% (1/2)	100% (2/2)	100% (4/4)	25% (1/4)	75% (3/4)	50% (1/2)	0% (0/2)	50% (1/2)
2	57% (8/14)	14% (2/14)	57% (8/14)	100% (6/6)	17% (1/6)	100% (6/6)	50% (3/6)	17% (1/6)	50% (3/6)	75% (3/4)	50% (2/4)	75% (3/4)
3	71% (12/17)	35% (6/17)	65% (11/17)	100% (1/1)	100% (1/1)	100% (1/1)	100% (3/3)	0% (0/3)	100% (3/3)	100% (1/1)	0% (0/1)	100% (1/1)
4	100% (6/6)	50% (3/6)	100% (6/6)	100% (2/2)	0% (0/2)	100% (2/2)	100% (1/1)	0% (0/1)	100% (1/1)	50% (1/2)	0% (0/2)	50% (1/2)

H: hepatocyte; N: nucleus; C: cytoplasm

immunohistochemically detected in the nucleus, mainly in nucleoli, and it has been thought to be involved in ribosomal biogenesis (Garrè et al., 1992; Penco et al., 2001). However, no relationships were found between Lf expression and clinico-pathological staging/grading as well as iron deposits in chronic hepatitis, irrespective to their aetiology. Interestingly, liver specimens from HBV hepatitis showed a constant Lf reactivity with the highest ID-score, whereas the evidence of Lf was variously represented in HCV as well as HDV chronic hepatitis; these data document a variable degree of Lf immunostaining in relation to the different viruses responsible for chronic hepatitis. However, it has been reported that bovine Lf prevents HCV infection in human cultured hepatocytes as well as the liver infection by hepatitis G virus, which is distantly related to HCV (Ikeda et al., 2000). In addition, a pilot study has suggested a role of Lf in decreasing the HCV viremia in patients with chronic hepatitis C (Tanaka et al., 1999); moreover, patients with chronic hepatitis C showed both decreased tear volume and tear lactoferrin concentrations (Abe et al., 1999). In our liver specimens from HCV hepatitis, the Lf nuclear immunoreactivity appeared to increase with the progression of the disease, while no relationship was noted in hepatitis with different aetiology. In addition, a significantly greater Lf expression was encountered in genotype 1 HCV, which is able to determine a viral infection less sensitive to interferon treatment as well as an increase in activity and fibrosis scores (Kanai et al., 1992; Takada et al., 1992; Fontaine et al., 2001). Since it has been suggested by *in vitro* experiments that the antiviral effect of Lf against HCV may be obtained through its binding to HCV E1 and E2 envelope proteins (Yi et al., 1997), the different Lf immunoreactivity that we observed in HCV specimens might be modulated by its interaction with the above mentioned viral proteins.

Another intriguing finding of our study was the

demonstration of Lf in hepatocytes from specimens with cryptogenic hepatitis: the immunophenotypic pattern of Lf was mainly appreciable in the cytoplasm of liver cells, similar to that observed in virus-induced hepatitis. This fact may suggest a role for Lf as an unspecific defensive agent in inflammatory liver diseases, even when silent viral infections can be excluded; however, our hypothesis is strongly supported by a similar evidence of Lf, elsewhere reported in other inflammatory lesions such as ulcerative colitis, primary sclerosing cholangitis, Crohn's disease (Peen et al., 1993, 1996; Baynes and Bezwoda, 1994; Britigan et al., 1994; Baveye et al., 1999) as well as autoimmune liver diseases (Ohana et al., 1998). On the other hand, the absence of Lf in our normal liver specimens further supports the involvement of Lf as one of the molecules modulating the inflammatory response.

In the light of our results, it might be hypothesized that affected hepatocytes either synthesize or take up Lf from the extracellular compartment in order to play an unspecific immunoregulatory activity. The immunohistochemical evidence of Lf in hepatocytes may be related to the production of this iron-binding protein in the cells themselves, although this interpretation should be tested by methods other than morphological analysis. Alternatively, the localisation of Lf in hepatocytes may not reflect an intracellular synthesis, being instead the consequence of defective or functionally impaired Lf receptors; in this way, the intracellular presence of Lf immunoreactivity may reveal the degree of transmembranous iron transfer.

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