Distribution of lactoferrin and 60/65 kDa heat shock protein in normal and inflamed human intestine and liver

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Abstract

Immunisation against the mycobacterial heat shock protein (hsp-65) has been proposed to lead to production of autoantibodies against human lactoferrin. Such antibodies occur in ulcerative colitis and in primary sclerosing cholangitis. This study analysed the distribution of hsp-65 and lactoferrin in biopsy specimens from patients with inflammatory bowel disease and primary sclerosing cholangitis and studied whether immunisation against mycobacterial hsp-65 resulted in production of antilactoferrin antibodies and vice versa. Polyclonal rabbit antihuman lactoferrin and monoclonal mouse anti-hsp-65 (ML30) were used for immunohistochemistry on biopsy specimens from patients with inflammatory bowel disease and primary sclerosing cholangitis. Rats were immunised against human lactoferrin and mycobacterial hsp-65 respectively. Antibody measurements were done by enzyme immunosorbent assays. It was found that lactoferrin and hsp-60/65 were not codistributed. Lactoferrin was found on vascular endothelium and in non-parenchymal liver cells both in inflamed and uninfamed tissues, but only in the hepatocytes of inflamed liver. ML30 reactivity was not inhibited by antilactoferrin antibodies. Rat anti-hsp-65 serum had no detectable antilactoferrin antibodies. In conclusion, antilactoferrin antibodies probably do not arise by immunisation against mycobacterial hsp-65. Both non-parenchymal cells and hepatocytes probably participate in clearance of lactoferrin. Endothelial exposure of lactoferrin may have pathogenic implications in diseases with antilactoferrin autoantibodies.

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'A red protein fraction' in bovine milk was discovered by Sørensen and Sørensen in 1939,¹ and in 1961 the designation lactoferrin was proposed by Blanc and Isliker.² Lactoferrin is a single chain 78 kDa glycoprotein consisting of two domains, each of which has an iron binding site.³ In human milk lactoferrin is a dominating protein, accounting for over 40% of the total protein content (>7 g/l) in colostrum, and about 30% (1.5–4 g/l) of the protein content in mature milk.⁴ Lactoferrin is secreted

not only by the mammary glands, but also by the pancreas, the kidneys, the mucosae of the endometrium, the seminal vesicles and by lachrymal, bronchial, and salivary glands.^{5,6} A receptor on the brush border of enterocytes binds lactoferrin, which may be of importance for iron absorption in the new born.⁷ Lactoferrin is probably also an important non-specific defence factor at mucosal surfaces, owing to its antibacterial properties.^{8–10} Furthermore, lactoferrin has powerful anti-inflammatory properties, for example, by preventing complement activation through inhibition of classic C3 convertase,¹¹ by preventing the formation of hydroxyl radicals,^{12,13} and by preventing bacterial lipopolysaccharide from activating polymorphonuclear neutrophil leucocytes (PMNL).¹⁴

Apart from production and secretion by exocrine glands, lactoferrin resides in the specific granules of PMNL,¹⁵ and becomes exocytosed upon activation of the cells.¹⁶ Normally, the plasma concentration of lactoferrin is very low, but it increases during inflammation owing to stimulation/degranulation of PMNL.^{17,18} Lactoferrin is released in iron free form, and by iron chelation and subsequent elimination by the reticuloendothelial system circulating lactoferrin may contribute to the hypsideraemia seen in acute and chronic inflammation.^{17,19} Raised concentrations of circulating lactoferrin can also induce immediate leucopenia²⁰ caused by increased PMNL adhesiveness to endothelial cells.²¹ Deposition of circulating lactoferrin on intestinal vascular endothelium may lead to recruitment of leucocytes and induction of tissue damage in inflammatory bowel disease.²²

Lactoferrin from different species differs considerably with respect to antigenicity.²³ Antibodies to bovine lactoferrin are common in serum of healthy subjects, whereas antihuman lactoferrin antibodies are not.²⁴ Autoantibodies to lactoferrin have been shown in patients with systemic rheumatoid vasculitis,²⁵ ulcerative colitis,^{24,26,27} primary sclerosing cholangitis,²⁴ and reactive arthritis.²⁸ Antilactoferrin antibodies belong to the family of antineutrophil cytoplasm antibodies (ANCA) – at indirect immunofluorescence microscopy on ethanol fixed human PMNL, antilactoferrin antibodies produce a perinuclear (P-ANCA) staining pattern.^{24,29}

Human lactoferrin has a short sequence homology with mycobacterial heat shock protein (hsp) 65, and it has been speculated that antilactoferrin autoantibodies may arise as the result of cross immunisation against

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mycobacterial hsp-65.^{30 31} Mycobacterial hsp-65 has more than 50% sequence homology with human hsp-60³² and immune responses against mycobacterial hsp-65 have attracted great interest as a possible pathogenic factor in experimental^{33–35} as well as in human autoimmune diseases, including rheumatoid arthritis and inflammatory bowel disease.^{36 37}

We were inspired to perform this study by the reports on immunological cross reactivity between mycobacterial hsp-65 and human lactoferrin, and by our own finding of antilactoferrin autoantibodies in ulcerative colitis and primary sclerosing cholangitis. We wished to investigate the distribution of lactoferrin in relation to the distribution of hsp-65 in histologically normal tissue specimens and in inflamed tissues from patients with inflammatory bowel disease and primary sclerosing cholangitis. We also aimed at performing experiments to see whether or not antilactoferrin antibodies have affinity for hsp-60/65 in human tissue sections, and if the induction of an anti-hsp-65 immune response results in cross immunisation against lactoferrin and vice versa.

Methods

Tissue specimens

Paraffin wax blocks of tissue specimens previously examined in the diagnostic routine were analysed. Light microscopically normal tissues consisted of small and large intestines as well as liver, and the pathological specimens considered of small intestine from five patients with Crohn's disease, large intestine from seven patients with ulcerative colitis, and a liver biopsy sample from a patient with primary sclerosing cholangitis. All tissue specimens had been fixed in buffered 4% formaldehyde. Four μ m sections were cut, deparaffinised, and rehydrated.

Antigens, immune serum samples, and antibodies

Human iron free milk lactoferrin (Sigma Chemical Co, MO) was used to immunise rabbits and rats, and to coat microtitre plates used for enzyme linked immunosorbent assays (ELISAs).

Recombinant mycobacterial hsp-65 (WHO/UDNP World Bank) was kindly provided by Dr Vidar Bakke, Centre for International Health, University of Bergen, Norway.

Monoclonal mouse antibodies (ML30 MoAb) recognising mycobacterial hsp-65 as well as human hsp-60 was the generous gift from Professor Juray Ivanyi, MRC Tuberculosis and Related Infections Unit, Royal Postgraduate Medical School, London.

Polyclonal antihuman lactoferrin antibodies were obtained by immunising rabbits with lactoferrin emulsified in Freund's incomplete adjuvant (Difco, Detroit, MI) as described elsewhere.²⁴ The same protocol was followed to immunise Lewis rats with human lactoferrin. Cross reactivity of the rabbit antihuman lactoferrin antibodies with human IgA was excluded by means of indirect immunofluorescence

microscopy. Thus, the rabbit antihuman lactoferrin antibodies failed to react with human IgA-antiendomysial antibodies bound to tissue sections of monkey oesophagus. Likewise, cross reactivity between rabbit antilactoferrin antibodies and secretory component was unlikely as no indirect immunofluorescence reaction was detected with secretory component on unfixed cryostat sections of rat liver and stomach, and considering the phylogenetically conserved structure of secretory component.³⁸

To obtain polyclonal anti-hsp-65 antibodies, rats were given repeated subcutaneous or intracutaneous injections of Freund's complete adjuvant (Difco). The rats were anaesthetised and bled by heart puncture.

Affinity purified biotinylated rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) and swine antirabbit immunoglobulins (Dakopatts) were used as secondary antibodies for immunohistochemistry. Rabbit antirat immunoglobulin antiserum conjugated with alkaline phosphatase (Dakopatts) was used for the ELISAs.

ELISA

Microtitre plates were coated with 10 μ g antigen/ml and incubated with rat immune serum diluted serially with phosphate buffered saline pH 7.6 containing 0.05% TWEEN-20. Details of the procedures have been described elsewhere.^{24 29}

Immunohistochemistry

Deparaffinised and rehydrated sections were treated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity, and incubated with normal rabbit serum or normal swine serum respectively before immunostaining to prevent non-specific background. Incubation with ML30 MoAb, diluted 1:1000–1:2000, or rabbit antilactoferrin antiserum, diluted 1:1000, was carried out for 60 minutes at room temperature. In an attempt to block the reactivity of ML30 MoAb by rabbit antilactoferrin and vice versa, the tissue sections were pre-incubated with the 'blocking' antibody for 60 minutes. The secondary biotinylated antibodies were diluted 1:2000 (swine) or 1:1000 (rabbit) and incubated with the tissue sections for 60 minutes. Avidin complexed with biotinylated peroxidase was used as the visualisation system with hydrogen peroxide as substrate and diaminobenzidine as chromogen.

Results

Immunisation

The serum of rabbits and rats immunised with lactoferrin had high values of antilactoferrin antibodies detectable at dilutions >1:10 000 judged by ELISA (not illustrated). Tested by ELISA, the rat antilactoferrin immune serum samples also had low values of antibodies binding hsp-65 at serum dilutions up to 1:16. Serum from rats given heat killed mycobacteria subcutaneously contained high concentrations

of anti-hsp-65 antibodies, but no detectable antibodies against human lactoferrin (not illustrated). The ML30 MoAb did not bind lactoferrin as tested by ELISA (not illustrated).

Distribution of hsp-60/65 and lactoferrin in human biopsy specimens

	hsp-65		hsp-65 (preincubation with antilactoferrin)	Lactoferrin	
	Staining	n/n tot*		Staining	Staining [†] n/n tot*
<i>Normal small intestine</i>					
Villus epithelium	+	(2/2)	+	++	(1/1)
Crypt cells	—	(2/2)	—	++	(1/1)
Intestinal glands	+	(2/2)	+	+	(1/1)
Stroma cells	—	(2/2)	—	+	(1/1)
Vascular endothelium	—	(2/2)	—	+	(1/1)
Inflammatory cells	(+)	(2/2)	—	+	(1/1)
<i>Crohn's disease, small intestine</i>					
Villus epithelium	—	(5/5)	—	+	(4/5)
Crypt cells	—	(5/5)	—	—	(4/5)
Intestinal glands	—	(5/5)	—	+	(3/5)
Stroma cells	—	(5/5)	—	+	(5/5)
Vascular endothelium	—	(5/5)	—	+	(5/5)
Inflammatory cells	—	(4/5)	—	+	(5/5)
<i>Normal large intestine</i>					
Surface epithelium	+	(2/2)	—	+	(1/1)
Glandular epithelium	+/—	(1+1—)	—	+	(1/1)
Stroma cells	—	(2/2)	—	+	(1/1)
Vascular endothelium	—	(2/2)	—	+	(1/1)
Inflammatory cells	—	(2/2)	—	+	(1/1)
<i>Ulcerative colitis, large intestine</i>					
Surface epithelium	—	(6/7**)	++	(+)	(5/5)
Glandular epithelium	—	(6/7**)	++	(+)	(4/5)
Stroma cells	—	(7/7)	—	+	(5/5)
Vascular endothelium	—	(7/7)	—	+	(5/5)
Inflammatory cells	—	(7/7)	—	+	(5/5)
<i>Normal liver</i>					
Hepatocytes	++	(2/2)	++	—	(1/1)
Non-parenchymal cells	—	(2/2)	—	++	(1/1)
Bile duct epithelium	+	(2/2)	+	+	(1/1)
<i>Primary sclerosing cholangitis</i>					
Hepatocytes	++	(1/1)	+	(+)	(1/1)
Non-parenchymal cells	—	(1/1)	—	++	(1/1)
Small bile ducts	+	(1/1)	(+)	+	(1/1)

*n=Number of positive or negative staining results; n tot=total number of examined biopsy specimens. ++=Strongly positive staining; +=positive staining; (+)=weakly positive staining; -=negative staining. †The biopsy specimen with positive hsp-staining (1/7) was used in the 'blocking' experiment.

Distribution of lactoferrin and hsp-60/65
The Table summarises the distributions of human lactoferrin and antigen accessible to ML30 MoAb recognising mycobacterial/human hsp-65/60. The results of the blocking experiments by preincubation with rabbit-antilactoferrin prior to incubation with and staining for hsp-60/65 are shown in the Table.
There was no strict codistribution of lactoferrin and hsp-60/65, and preincubation of the tissue sections with rabbit-antilactoferrin did not affect the access of antigen binding by ML30 MoAb recognising hsp-60/65.
The staining patterns of normal and inflamed tissues with respect to lactoferrin was essentially the same. Lactoferrin was shown intracellularly in both enterocytes and mucosal glands, although the staining was more intense in normal tissue specimens (Fig 1a, b).
Furthermore, the vascular endothelium of small vessels were positively stained for lactoferrin in normal as well as in inflamed tissue (not illustrated). Also the staining patterns achieved with ML30 MoAb were essentially the same in normal and inflamed tissues with a few notable exceptions: the villus epithelium of the small intestine was only stained in the normal tissues (Fig 1c), but not in Crohn's disease (Fig 1d). Similarly, the surface epithelium of the large intestine was positively stained in normal tissue specimens, but not in ulcerative colitis (not illustrated).
Staining for lactoferrin was seen intracellularly in non-parenchymal liver cells, but not in the hepatocytes of normal liver (Fig 2a). Positive staining for lactoferrin was seen also in the bile duct epithelial cells (not illustrated). In

Figure 1: Strongly positive intracytoplasmic staining for lactoferrin in the enterocytes of histologically normal small bowel (a) and weaker staining in Crohn's disease (b). Positive lactoferrin staining is shown also on the luminal surface of the enterocytes and in infiltrating polymorphonuclear leucocytes. Positive staining for hsp-60/65 is evident within the enterocytes of histologically normal small bowel (c), whereas apparently no staining is seen in a small bowel biopsy specimen from a patient with Crohn's disease (d). Original magnifications $\times 50-80$.

Figure 2: Negative staining for lactoferrin in the hepatocytes of histologically normal liver (a), and slightly positive lactoferrin staining in primary sclerosing cholangitis (b). In both instances non-parenchymal liver cells are positively stained. The positive staining patterns for hsp-60/65 were identical in histologically normal liver (c) and primary sclerosing cholangitis (d). Original magnification $\times 200$.

the liver specimen from a patient with primary sclerosing cholangitis, faint lactoferrin staining was detected also in the hepatocytes, apart from the intense staining of non-parenchymal cells (Fig 2b). Lactoferrin was always seen in infiltrating PMNL. Figure 2c and d show the identical staining patterns for hsp-60/65 in a normal liver biopsy specimen and in the liver of a patient with primary sclerosing cholangitis.

Discussion

Lactoferrin, an iron binding protein with antimicrobial, anti-inflammatory, and immunoregulatory properties, normally occurs in high concentrations in mucosal secretions and in milk (for reviews, see^{7 39 40}). It is also released by activated polymorphonuclear granulocytes resulting in raised plasma concentrations during inflammation.^{17 18} Apart from its antiphlogistic actions, circulating lactoferrin may also act in a proinflammatory way by recruiting leucocytes and increasing their attachment to vessel walls by interaction with endothelial P-selectin.^{20–22} The finding in this study of lactoferrin accessible to antilactoferrin antibodies on vascular endothelium may hypothetically be of pathogenic relevance in diseases with circulating antilactoferrin autoantibodies, like ulcerative colitis, primary sclerosing cholangitis, and systemic rheumatoid vasculitis.^{24–27} This speculation is further substantiated by the fact that intact immunoglobulin G antilactoferrin antibodies bound to vascular endothelial cells stimulate granulocytes to produce free oxygen radicals *in vitro* (Peen, *et al*, unpublished data). Hypothetically, antilactoferrin autoantibodies may

also promote inflammation by counteracting the antiphlogistic properties of lactoferrin, for example, the production of hydroxyl radicals.¹³

The origin of antilactoferrin autoantibodies is not known. Structural homology between lactoferrin and mycobacterial hsp-65 has been shown,^{30 31} and lactoferrin has been reported to cause T cell proliferation in arthritic rats immunised against mycobacteria.³² Based upon these findings it was speculated that an immune response directed against human lactoferrin could be initiated by primary immunisation against mycobacterial hsp-65, and that a humoral or cellular antilactoferrin immune response, or both, may be a pathogenic factor in the development of chronic autoimmune arthritis.^{30–32} This seems highly unlikely, however, in the light of the results of Hajeer and Bernstein.⁴¹ Indeed, they found low concentrations of anti-hsp-65 antibodies in serum of rabbits immunised with lactoferrin together with either Freund's complete or incomplete adjuvant. Affinity purified antilactoferrin antibodies, however, did not bind hsp-65, and anti-hsp-65 activity was not inhibited by lactoferrin.⁴¹ Furthermore, anti-hsp-65 antibodies were found also in antisera against human serum albumin.⁴⁰ In this study we confirmed that very low serum concentrations of anti-hsp-65 antibodies were found in rats immunised with lactoferrin in incomplete Freund's adjuvant, whereas no lactoferrin binding was seen testing polyclonal rat anti-hsp-65 antibodies in an ELISA. Furthermore, polyclonal high titred rabbit antilactoferrin immune serum did not inhibit the binding of monoclonal mouse anti-hsp-65 to human biopsy specimens.

The finding of lactoferrin in the non-parenchymal liver cells of human biopsy specimens probably reflects lactoferrin clearance from the circulation, as lactoferrin is not produced by mononuclear phagocytes.¹⁷ Positive staining for lactoferrin in the non-parenchymal liver cells, but not in the hepatocytes, is interesting with respect to previously published contradictory results on the hepatic handling of circulating lactoferrin. Although the liver is known to be the important site of lactoferrin removal from the blood, conflicting reports have been published concerning the main type of liver cell involved. Some investigators have reported that the non-parenchymal liver cells are most important,^{17 42-44} whereas others claim that the hepatocytes alone are responsible for the clearance of circulating lactoferrin.⁴⁵⁻⁴⁷ Debanne *et al*⁴⁸ showed that non-parenchymal cells as well as hepatocytes can be involved, and these findings were recently confirmed in an experimental study from our laboratory.⁴⁹ The absence of hepatocyte lactoferrin staining in histologically normal human liver, which has also been reported by others,⁵⁰ could result from an insensitive method rather than the absence of lactoferrin in these cells. In inflammatory conditions, the concentrations of circulating lactoferrin are higher.^{17 18} Therefore the faint staining for lactoferrin also in the hepatocytes of a patient with primary sclerosing cholangitis probably reflects clearance of circulating lactoferrin also by this cell type.

A physiological function of lactoferrin may be to facilitate iron absorption by receptor mediated binding of holo-lactoferrin by enterocytes followed by transcytosis and iron release.^{7 51}

Therefore, the finding of lactoferrin in enterocytes in this study could speculatively reflect endocytosed external protein.

We conclude that lactoferrin is distributed similarly in biopsy specimens of liver and intestine from normal and inflamed tissue. In both instances the non-parenchymal liver cells were positively stained, whereas signs of intracellular lactoferrin in the hepatocytes were seen only in liver sections from patient with primary sclerosing cholangitis. This supports the view that both non-parenchymal liver cells and hepatocytes participate in the clearance of circulating lactoferrin. A monoclonal antibody specific for mycobacterial hsp-65/human hsp-60 (ML30 MoAb) did not recognise the same tissue structures as polyclonal antibodies against human lactoferrin, and the tissue reactivity of ML30 MoAb was not inhibited by antilactoferrin antibodies. This taken together with the absence of antilactoferrin antibodies in rat immune serum containing high values of anti-hsp-65 antibodies, contradicts the hypothesis that antilactoferrin antibodies arise as the result of immunisation against mycobacterial hsp-65.

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