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

E Peen, S Eneström, T Skogh

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 nonparenchymal 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 nonparenchymal cells and hepatocytes probably participate in clearance of lactoferrin. Endothelial exposure of lactoferrin may have pathogenic implications in diseases with antilactoferrin autoantibodies.

(Keywords: lactoferrin, mycobacterial heat shock protein 65, inflammation, autoimmune disease.

'A red protein fraction' in bovine milk was discovered by Sørensen and Sørensen in 1939, and in 1961 the designation lactoferrin was proposed by Blanc and Islakr. Lactoferrin is a single chain 78 kDa glycoprotein consisting of two domains, each of which has an iron binding site. 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. Lactoferrin is secreted not only by the mammary glands, but also by the pancreas, the kidneys, the mucosa of the endometrium, the seminal vesicles and by lachrymal, bronchial, and salivary glands. A receptor on the brush border of enterocytes binds lactoferrin, which may be of importance for iron absorption in the new born. Lactoferrin is probably also an important nonspecific defence factor at mucosal surfaces, owing to its antibacterial properties. Furthermore, lactoferrin has powerful anti-inflammatory properties, for example, by preventing complement activation through inhibition of classic C3 convertase, by preventing the formation of hydroxyl radicals, and by preventing bacterial lipopolysaccharide from activating polymorphonuclear neutrophil leucocytes (PMNL). Apart from production and secretion by exocrine glands, lactoferrin resides in the specific granules of PMNL and becomes exocytosed upon activation of the cells. Normally, the plasma concentration of lactoferrin is very low, but it increases during inflammation owing to stimulation/degranulation of PMNL. Lactoferrin is released in iron free form, and by iron chelation and subsequent elimination by the reticuloendothelial system circulating lactoferrin may contribute to the hypoferremia seen in acute and chronic inflammation. Raised concentrations of circulating lactoferrin can also induce immediate leucopenia caused by increased PMNL adhesiveness to endothelial cells. Deposition of circulating lactoferrin on intestinal vascular endothelium may lead to recruitment of leucocytes and induction of tissue damage in inflammatory bowel disease. Lactoferrin from different species differs considerably with respect to antigenicity. Antibodies to bovine lactoferrin are common in serum of healthy subjects, whereas antihuman lactoferrin antibodies are not. Autoantibodies to lactoferrin have been shown in patients with systemic rheumatoid vasculitis, ulcerative colitis, primary sclerosing cholangitis, and reactive arthritis. 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. 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...
mycobacterial hsp-65. Mycobacterial hsp-65 has more than 50% sequence homology with human hsp-60 and immune responses against mycobacterial hsp-65 have attracted great interest as a possible pathogenic factor in experimental as well as in human autoimmune diseases, including rheumatoid arthritis and inflammatory bowel disease.

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 μm 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 immunosorbert 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. 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-antienzyme 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.

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 antimouse immunoglobulins (Dakopatts, Glostrup, Denmark) and swine antirabbit immunoglobulins (Dakopatts) were used as secondary antibodies for immunohistochemistry. Rabbit antirat immunoglobulin antisera conjugated with alkaline phosphatase (Dakopatts) was used for the ELISAs.

ELISA
Microtitre plates were coated with 10 μg 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.

Immunohistochemistry
Decarboxinised 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 diamonobenzidine 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

<table>
<thead>
<tr>
<th></th>
<th>hsp-60/65</th>
<th>hsp-65 (preincubation with anti-lactoferrin)</th>
<th>Lactoferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staining</td>
<td>n/n total*</td>
<td>Staining</td>
</tr>
<tr>
<td>Normal small intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus epithelium</td>
<td>+ (2/2)</td>
<td>+</td>
<td>++ (1/1)</td>
</tr>
<tr>
<td>Crypt cells</td>
<td>– (2/2)</td>
<td></td>
<td>+ (1/1)</td>
</tr>
<tr>
<td>Intestinal glands</td>
<td>+ (2/2)</td>
<td>+</td>
<td>+ (1/1)</td>
</tr>
<tr>
<td>Stroma cells</td>
<td>– (2/2)</td>
<td>+</td>
<td>+ (1/1)</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td>– (2/2)</td>
<td>+</td>
<td>+ (1/1)</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>(+) (2/2)</td>
<td>+</td>
<td>+ (1/1)</td>
</tr>
<tr>
<td>Crohn's disease, small intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Villus epithelium</td>
<td>– (5/5)</td>
<td>+</td>
<td>(4/5)</td>
</tr>
<tr>
<td>Crypt cells</td>
<td>– (5/5)</td>
<td></td>
<td>(4/5)</td>
</tr>
<tr>
<td>Intestinal glands</td>
<td>– (5/5)</td>
<td>+</td>
<td>(3/5)</td>
</tr>
<tr>
<td>Stroma cells</td>
<td>– (5/5)</td>
<td>+</td>
<td>(5/5)</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td>– (5/5)</td>
<td>+</td>
<td>(5/5)</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>– (4/5)</td>
<td>+</td>
<td>(5/5)</td>
</tr>
<tr>
<td>Normal large intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface epithelium</td>
<td>+ (2/2)</td>
<td>+</td>
<td>(1/1)</td>
</tr>
<tr>
<td>Glandular epithelium</td>
<td>+/- (1+1-)</td>
<td></td>
<td>(1/1)</td>
</tr>
<tr>
<td>Stroma cells</td>
<td>– (2/2)</td>
<td>+</td>
<td>(1/1)</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td>– (2/2)</td>
<td>+</td>
<td>(1/1)</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>– (2/2)</td>
<td>+</td>
<td>(1/1)</td>
</tr>
<tr>
<td>Ulcerative colitis, large intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface epithelium</td>
<td>– (6/7***)</td>
<td>+†</td>
<td>+ (5/5)</td>
</tr>
<tr>
<td>Glandular epithelium</td>
<td>– (6/7***)</td>
<td>+†</td>
<td>+ (4/5)</td>
</tr>
<tr>
<td>Stroma cells</td>
<td>– (7/7)</td>
<td>+</td>
<td>+ (5/5)</td>
</tr>
<tr>
<td>Vascular endothelium</td>
<td>– (7/7)</td>
<td>+</td>
<td>+ (5/5)</td>
</tr>
<tr>
<td>Inflammatory cells</td>
<td>– (7/7)</td>
<td>+</td>
<td>+ (5/5)</td>
</tr>
<tr>
<td>Normal liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>+ (2/2)</td>
<td>+</td>
<td>(1/1)</td>
</tr>
<tr>
<td>Non-parenchymal cells</td>
<td>– (2/2)</td>
<td></td>
<td>+† (1/1)</td>
</tr>
<tr>
<td>Bile duct epithelium</td>
<td>+ (2/2)</td>
<td>+</td>
<td>+ (1/1)</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>+ (1/1)</td>
<td>+</td>
<td>(1/1)</td>
</tr>
<tr>
<td>Non-parenchymal cells</td>
<td>– (1/1)</td>
<td></td>
<td>+† (1/1)</td>
</tr>
<tr>
<td>Small bile ducts</td>
<td>+ (1/1)</td>
<td>+</td>
<td>+ (1/1)</td>
</tr>
</tbody>
</table>

* = Number of positive or negative staining results; n = 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.

Figure 1: Strongly positive intracytoplasmatic 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 leukocytes. 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 ×50–80.
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[1][2][3][4][5]). It is also released by activated polymorphonuclear granulocytes resulting in raised plasma concentrations during inflammation.[6][7][8] 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.[9][10][11][12] 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.[13][14] 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.[15]

The origin of antilactoferrin autoantibodies is not known. Structural homology between lactoferrin and mycobacterial hsp-65 has been shown,[16][17][18] and lactoferrin has been reported to cause T cell proliferation in arthritic rats immunised against mycobacteria.[19] Based upon these findings it was speculated that an immune response directed against human lactoferrin could be initiated by primary immunisation against mycobacteral 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.[20][21][22] This seems highly unlikely, however, in the light of the results of Hajeer and Bernstein.[23] 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.[24] Furthermore, anti-hsp-65 antibodies were found also in antisera against human serum albumin.[25] 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 titre 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.\textsuperscript{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,\textsuperscript{17 42-44} whereas others claim that the hepatocytes alone are responsible for the clearance of circulating lactoferrin.\textsuperscript{45-47} Debanne et al.\textsuperscript{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.\textsuperscript{49} The absence of hepatocyte staining for lactoferrin in histologically normal human liver, which has also been reported by others,\textsuperscript{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.\textsuperscript{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 to enterocytes followed by transcytosis and iron release.\textsuperscript{71} 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 intra-cellular 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 mononuclear antibody specific for mycobacteriastatic hsp-65/47 (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 anti-lactoferrin antibodies. This taken together with the absence of anti-lactoferrin antibodies in rat immune serum containing high values of anti-hsp-65 antibodies, contradicts the hypothesis that anti-lactoferrin antibodies arise as the result of immunisation against mycobacterial hsp-65.\textsuperscript{6}

We thank Professor Juraj Ivanyi for generously providing monoclonal antibodies to mycobacterial hsp-65 (MoAb ML30), and Dr. Alex Karlsen for helpful cooperation.

The study was financed by grants from the Norwegian Research Council (project 344.92/014), the Swedish Medical Research Council (project B94-16X-10390-02B), King Gustaf V's 80-year foundation, Nanna Swartz foundation, and the Swedish Association Against Rheumatism, ÖLÄ Research Foundation, NORDFA, and research foundations at the University Hospital of Linköping. The technical assistance of Mrs Anne Nilsson, Mrs Laila Mentzoni, and Mrs Helene Wilhelmsson is acknowledged.


42. Retegui LA, Moguilevsky N, Castracane CP, Masson PL. Uptake of lactoferrin by the liver. I. Role of the reticuloendothelial system as indicated by blockade experiments. Lab Invest 1984; 50: 323-8.


Distribution of lactoferrin and 60/65 kDa heat shock protein in normal and inflamed human intestine and liver.
E Peen, S Eneström and T Skogh

Gut 1996 38: 135-140
doi: 10.1136/gut.38.1.135

Updated information and services can be found at:
http://gut.bmj.com/content/38/1/135

Email alerting service

These include:
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections

Articles on similar topics can be found in the following collections

Pancreas and biliary tract (1949)
Ulcerative colitis (1113)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/