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Glycosylated and unglycosylated human lactoferrins both bind iron and show identical affinities towards human lysozyme and bacterial lipopolysaccharide, but differ in their susceptibilities towards tryptic proteolysis

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We studied the role of N-glycosylation of human lactoferrin (hLF) with respect to properties that are relevant to its antibacterial and anti-inflammatory activities. A human kidney-derived 293(S) cell line that constitutively expresses recombinant hLF (rhLF) was produced. The reactivity towards various antibodies of rhLF that had been expressed in the absence or presence of tunicamycin (which blocks N-linked glycosylation) did not differ from that of natural (human milk-derived) hLF. Cation-exchange chromatography and N-terminal protein sequencing showed identical cationic properties and an intact N-terminal sequence for rhLF and natural hLF. SDS/PAGE of rhLF expressed in the presence of tunicamycin revealed a protein with the same M_r as that of enzymically deglycosylated natural hLF. Both glycosylated and unglycosylated rhLF appeared to be completely saturated with iron. The affinity of natural hLF, glycosylated and non-glycosylated rhLF for both human lysozyme (K_d 4.5×10^{-8} M) and bacterial lipopolysaccharide did not differ. SDS/PAGE of hLF species subjected to trypsin indicated that unglycosylated rhLF was much more susceptible to degradation. Furthermore, this analysis suggests that N-glycosylation heterogeneity in natural hLF and rhLF resides in the C-lobe. Thus our results provide no argument for differential antibacterial and/or anti-inflammatory activity of natural and (glycosylated) rhLF and suggest that a major function of glycosylation in hLF is to protect it against proteolysis.

INTRODUCTION

Lactoferrin (LF) is a single-chain metal-binding glycoprotein of M_r 80000 that belongs to the transferrin family [1]. LF consists of two lobes of high sequence similarity designated the N- and the C-lobe. Each lobe can bind a ferric ion while simultaneously incorporating one bicarbonate ion [1]. LF is present in all mucosal secretions, including milk, tears, vaginal secretion and seminal fluid. It is also present in the specific granules of polymorphonuclear leucocytes [2]. The physiological significance of LF is as yet not understood. A variety of biological actions of LF have been observed *in vitro* (for a review, see [2]). The bacteriostatic activity towards a large variety of potentially pathogenic bacteria [3] resides in its ability to bind iron with high affinity. Its bactericidal activity [4] is mediated through direct binding of LF via its strongly positively charged N-terminal portion [5] to outer-membrane components such as lipopolysaccharide (LPS) [6,7]. Synergy between the antibacterial action of hLF and human lysozyme (hLZ) has been reported [6]. The anti-inflammatory activities of LF include inhibition of the formation of hydroxyl radicals (by scavenging free iron) [8], inhibition of cytokine production [9,10] and binding to LPS [7], which is an important inflammatory mediator. LF has also been shown to promote the growth of intestinal cells [11] as well as that of *Bifidobacterium* species [12], which are the predominant bacteria of the intestinal flora of healthy breast-fed infants. Specific LF receptors have been isolated from intestinal cells [13,14], lymphocytes [15] and bacteria [16]. On the basis of LF activities observed *in vitro*, the main physiological role of LF may be to inhibit the growth of pathogenic bacteria and to reduce inflammatory responses at mucosal surfaces. Another role of LF may be to mediate transport of iron, e.g. from the mother to the newborn [17].

Human LF contains three possible N-glycosylation sites, two of which have been shown to be glycosylated (asparagine-138 and -479, located in the N- and C-lobe respectively) [18]. N-linked glycosylation of hLF is of the sialyl-*N*-acetyl-lactosaminic type, with a fucose residue α1-6-linked to the internal *N*-acetylglucosamine [19]. Glycosylation microheterogeneity of hLF from milk is caused by a variable number of sialic acid residues and by the possibility of α1-3-fucosylation of the external *N*-acetylglucosamine. It is not clear whether this hLF glycosylation microheterogeneity accounts for the presence of two hLF protein bands on SDS/PAGE [20,21].

The role of the glycosylation in hLF function is not understood. Several studies using enzymically (partially or completely) deglycosylated hLF did not reveal any apparent biological role of glycosylation in binding of hLF to human intestinal and to bacterial receptors [14,22], nor in its clearance from the mouse circulation [23]. However, one study showed that deglycosylation impaired binding of iron by hLF [24].

Here we describe the expression of glycosylated and unglycosylated recombinant hLF (rhLF) in human kidney-derived 293(S) cells. We found that glycosylated and unglycosylated

Abbreviations and definitions used: LF, lactoferrin; hLF, human lactoferrin; natural hLF, hLF from human milk; iron-saturated natural hLF, natural hLF that has completely been saturated with iron *in vitro*; rhLF, recombinant human lactoferrin; LPS, lipopolysaccharide; hLZ, human lysozyme; mAb, monoclonal antibody; SBTI, soybean trypsin inhibitor.

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rhLF were both saturated with iron and did not differ with respect to their affinities for LPS and hLZ. However, unglycosylated rhLF was much more susceptible to tryptic proteolysis than its glycosylated counterpart.

MATERIALS AND METHODS

Reagents

CNBr-activated Sepharose 4B, S Sepharose and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Human LF isolated from human milk was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or was obtained as described below. Soybean trypsin inhibitor (SBTI, type I-S) and bovine pancreatic trypsin (type III-S), tunicamycin and polyclonal rabbit anti-hLF antiserum were also purchased from Sigma. Anti-hLF monoclonal antibodies (mAbs) 13.17 [25,26] and 13.19, which recognize different hLF epitopes, were generously given by Dr. C. E. van der Schoot of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands). Polyclonal bovine anti-hLF antiserum was obtained after repeated intramuscular injections with hLF purified from human milk (see below); specific antibody was purified by affinity chromatography on hLF–Sepharose as described in [26] and conjugated with horseradish peroxidase according to standard procedures [27]. All the cell-culture reagents were from Gibco (Paisley, Renfrewshire, Scotland, U.K.). [^{35}S]Methionine, ^{125}I and ^{125}I-labelled Bolton–Hunter reagent were from Amersham (Bucks., U.K.).

Purification of natural hLF, hLF and hLZ

Human LF was purified from fresh human milk by cation-exchange chromatography on S Sepharose. Human milk to which sodium phosphate, pH 7.5 (20 mM final concn.), NaCl (0.4 M), and Tween-20 (0.02%, w/v) had been added, was batch-wise incubated with S Sepharose. After 4 h, the S Sepharose was washed with 20 mM sodium phosphate/0.4 M NaCl/0.02% Tween-20, pH 7.5. The S Sepharose was poured into a column, and hLF was eluted with 20 mM sodium phosphate/1.0 M NaCl, pH 7.5. Human LF was dialysed against 0.15 M NaCl and stored at −70 °C. Absorption measurements at 280 and 465 nm revealed that purified hLF (hereafter called 'natural hLF') was only 3.5% saturated. $A_{280}^{1\%}$ values of 11 and 14 were used for unsaturated and iron-saturated hLF respectively and an $A_{465}^{1\%}$ value of 0.58 for iron-saturated hLF [17].

Recombinant hLF (rhLF) produced by 293(S) cells (see below) was purified essentially as described for natural hLF.

Human lysozyme was purified as follows. To human milk depleted from hLF by incubation with S Sepharose (see above), 3 vol. of 20 mM sodium phosphate/0.02% Tween-20, pH 7.5, were added. This diluted milk was batchwise incubated with S Sepharose. S Sepharose was poured into a column, washed with 20 mM sodium phosphate/0.1 M NaCl, pH 7.5, and eluted with 20 mM sodium phosphate/0.5 M NaCl, pH 7.5. The S Sepharose eluate was subsequently filtered through a Sephacryl S-200 column in 10 mM sodium phosphate/0.15 M NaCl, pH 7.4 (PBS). Fractions containing hLZ (indicated by an hLZ-specific RIA) were pooled. SDS/PAGE of 10 μg protein samples showed a single protein band of M_r 14000.

Saturation of hLF with iron

Natural hLF was saturated with iron at 20 °C with a freshly prepared FeNTA solution [9.9 mM ferric nitrate (Sigma) and 8.5 mM nitrilotriacetic acid (disodium salt) (Sigma) in water, adjusted to pH 7.0 by adding solid $NaHCO_3$]. FeNTA was added to hLF to achieve a molar ratio of iron to hLF of 4:1 in the incubation mixture, i.e. a 2-fold molar excess of iron per iron-binding site. Iron-saturated hLF, hereafter called 'iron-saturated natural hLF', was then dialysed against 0.15 M NaCl.

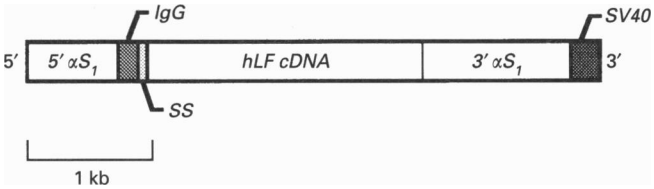

Figure 1 Schematic representation of expression vector paS1/hLF

Vector sequences: *5′ αS1*, 0.7 kb 5′ bovine αS_1 casein flanking/promoter sequence (nucleotides −680 to +150 of the αS_1 casein gene [46]); *IgG*, human IgG splice acceptor [47]; *ss*, bovine αS_1 casein signal sequence (nucleotides 1435–1483 of the αS_1 casein gene [46]); *hLF cDNA*, nucleotides 352–2428 [28]; *3′ αS1*, 1.2 kb 3′ bovine αS_1 casein sequence (nucleotides 16309–17455 of the αS_1 casein gene [46]); *SV40*, simian-virus-40 poly(A) signal.

hLF expression vector, transfection and cell culture conditions

A schematic representation of the vector pαS1/hLF, which contains a full-length hLF cDNA [28], is shown in Figure 1. The construction of this vector is considered beyond the scope of this paper (details are available from J. H. N. on request). Human kidney 293(S) cells [A.T.C.C. (American Type Culture Collection) CRL 1573] were co-transfected with paS1/hLF and RSVNeo by a calcium phosphate co-precipitation method [29]. Cells were cultured in Dulbecco's modified Eagle medium supplemented with 5% (v/v) fetal-calf serum, L-glutamine (3 μg/ml), penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37 °C in a humidified CO_2/air (1:19) incubator. After transfection, 800 μg/ml of G418 (Geneticin) was included in the culture medium to select for plasmid-containing cells. Subcloning of rhLF-expressing cells (as determined by an RIA for hLF) was performed by repeated limiting dilutions. The cells of a clone designated hLFNeo were grown to about 80% confluency in culture medium without G418. After two washes with PBS, fresh serum-free culture medium was added. To inhibit N-linked glycosylation [30], serum-free medium containing 5 μg/ml tunicamycin was used. Supernatants were harvested after 3 days of culture.

Metabolic labelling of rhLF with [^{35}S]methionine

The hLFNeo cells were grown to confluency and washed twice with PBS. Cells were subsequently starved for methionine for 30 min in methionine-free RPMI medium supplemented with L-glutamine, penicillin, streptomycin and FCS before [^{35}S]methionine was added. In some cases, 5 μg/ml tunicamycin was added to the starvation medium. After 16 h labelling, supernatants were harvested and cleared by centrifugation.

SDS/PAGE analysis

Non-reduced SDS/7.5% (w/v)-PAGE was performed using the Laemmli buffer system [31]. In some cases, samples were boiled for 5 min in non-reducing SDS sample buffer before SDS/PAGE analysis to achieve denaturation and concomitant desaturation of hLF [20]. Deglycosylation of hLF with N-glycosidase F (Boehringer Mannheim, Mannheim, Germany) was performed as recommended by the manufacturer. Supernatants of [^{35}S]-

methionine-labelled cells were incubated for 2 h with anti-hLF mAb 13.19 coupled to Sepharose. The beads were then washed twice with PBS/0.5 M NaCl/0.1 % Tween-20, followed by two washes with PBS/0.1 % Tween-20. [^{35}S]rhLF was eluted from the Sepharose by incubation for 5 min in non-reducing SDS sample buffer. Material containing released ^{35}S radioactivity (10000 c.p.m.) was subjected to SDS/PAGE. Gels were fixed and treated with En^{3}Hancer (du Pont) before autoradiography using Kodak-X Omat AR film.

RIAs

A quantitative RIA for hLF with mAb 13.17 coupled to Sepharose was performed essentially as described in [26]. Briefly, mAB 13.17–Sepharose suspension was incubated by head-over-head rotation with test samples. Sepharose beads were then washed and the hLF bound to Sepharose was detected by incubation with polyclonal rabbit ^{125}I-anti-hLF antiserum. After washing, Sepharose-bound radioactivity was measured. Results were expressed as percentage binding of the total amount of labelled antibodies added. The same procedure was followed in RIAs in which hLZ–Sepharose (a 0.5 ml suspension of 2.3 μg of hLZ coupled to 1 mg of CNBr-activated Sepharose 4B, in PBS/0.1 % Tween-20/0.02 % NaN_3) or LPS–Sepharose (a 0.5 ml suspension of Re595 LPS coupled to aminohexyl-Sepharose in PBS/0.1 % Tween-20/0.02 % NaN_3) was used instead of mAb 13.17–Sepharose. The specificity of hLF interaction with solid-phase hLZ and LPS was studied by RIAs in which glycine–Sepharose (a 0.5 ml suspension of 1.9 mg of glycine coupled to 1.0 mg of Sepharose) was used.

Tryptic proteolysis of hLF

Supernatants of hLFNeo cells, cultured in serum-free medium with or without tunicamycin, as well as purified natural hLF, were diluted [in serum-free medium conditioned by non-transfected 293(S) cells] to contain hLF at 0.3 μg/ml. These samples had trypsin (final concentration 200 μg/ml) added to them and they were incubated at 37 °C. At various time-points, 0.1 ml samples were drawn, and digestion was stopped by the addition of a 10-fold molar excess of SBTI. Human LF from these samples was immunoprecipitated by incubation with purified bovine anti-hLF antibodies coupled to Sepharose. Beads were then washed three times with PBS/0.1 % Tween-20, and hLF was dissociated into non-reducing SDS sample buffer by incubation at 100 °C for 5 min. The samples were subjected to SDS/12.5 %-PAGE, and proteins from the gel were transferred on to nitrocellulose as described in [32]. Blots were incubated in 100 mM Tris/HCl/150 mM NaCl (pH 7.5)/0.1 % Tween-20 (TTBS) containing 3 % BSA. Immunoblotting was performed with peroxidase-labelled bovine anti-hLF antibodies diluted in TTBS. After four washes with TTBS, hLF protein bands on blots were revealed by chemoluminiscence detection (ECL, Amersham International).

RESULTS

Expression of rhLF by 293(S) cells

The hLF cDNA-based expression vector pαS1/hLF (see Figure 1) was stably transfected into 293(S) cells. Subcloning of the transfected cells resulted in several lines expressing rhLF. The cell line designated hLFNeo, expressing rhLF up to 0.6 μg/ml, was used for further experiments. Figure 2 shows dose–response curves of natural hLF and of rhLF expressed in the presence or absence of tunicamycin in the RIA for hLF. The identical slopes and maximal responses in the RIA indicate that antigenic determinants for monoclonal and polyclonal anti-hLF antibodies in natural and rhLF species are equally well accessible. Natural and rhLF species thus appear immunologically identical. By reference to natural hLF (5 μg/ml), we calculated that the sample of conditioned medium of hLFNeo cultured with tunicamycin contained 0.13 μg/ml hLF, whereas that of cells cultured without tunicamycin contained 0.50 μg/ml (Figure 2).

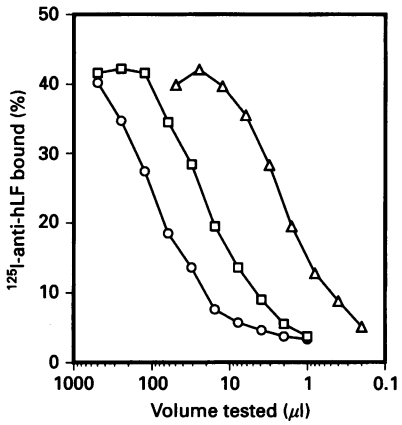

Figure 2 Radioimmunoassay for hLF antigen

Two-fold serial dilutions of Sigma hLF (5 μg/ml; △) and conditioned medium of hLFNeo, cultured either with (○) or without (□) tunicamycin, were incubated with anti-hLF mAb 13.17 coupled to Sepharose as described in the Materials and methods section. Bound hLF was detected by subsequent incubation with polyclonal ^{125}I-anti-hLF antibodies. Results are expressed as percentage binding of the ^{125}I-anti-hLF antibodies added. The volume of the experimental sample tested (μl) is indicated on the abscissa.

Purification and N-terminal-sequence analysis of natural and rhLF

During the course of hLF purification studies we found that several lots of purified hLF preparations from various commercial suppliers eluted in three peaks from a Mono S column (Figure 3a). N-terminal protein sequencing indicated that the hLF eluted at 0.7 M NaCl represented native hLF, whereas the hLF eluted at 0.6 and 0.5 M NaCl represented hLF from which two (GR) and three (GRR) N-terminal residues respectively had been removed by limited proteolysis.

Natural hLF and rhLF were isolated from fresh human milk and serum-free conditioned medium, respectively by cation-exchange chromatography on S Sepharose. Figure 3 shows the Mono S elution pattern of purified human milk-derived hLF (Figure 3b), purified rhLF (Figure 3c), and purified rhLF expressed in the presence of tunicamycin (Figure 3d). All three profiles show a major protein peak that was eluted at about 0.7 M NaCl which, according to our hLF-specific RIA, corresponds to hLF. This experiment shows that natural hLF and rhLF have the same cationic properties. N-terminal-sequence analysis revealed that the natural and recombinant hLF had identical sequences (Figures 3b, 3c and 3d). This indicates that preparations were pure and that no proteolysis had occurred. The sequence of rhLF was identical with that predicted on the basis of the DNA sequence, indicating that the bovine αS_1-casein signal sequence had correctly and completely been removed in 293(S) cells to yield mature rhLF. Material which was eluted at 0.65 and 0.8 M NaCl on Mono S chromatography of rhLF from tunicamycin-treated hLFNeo cells (Figure 3d) did not show any response in the RIA for antigenic hLF.

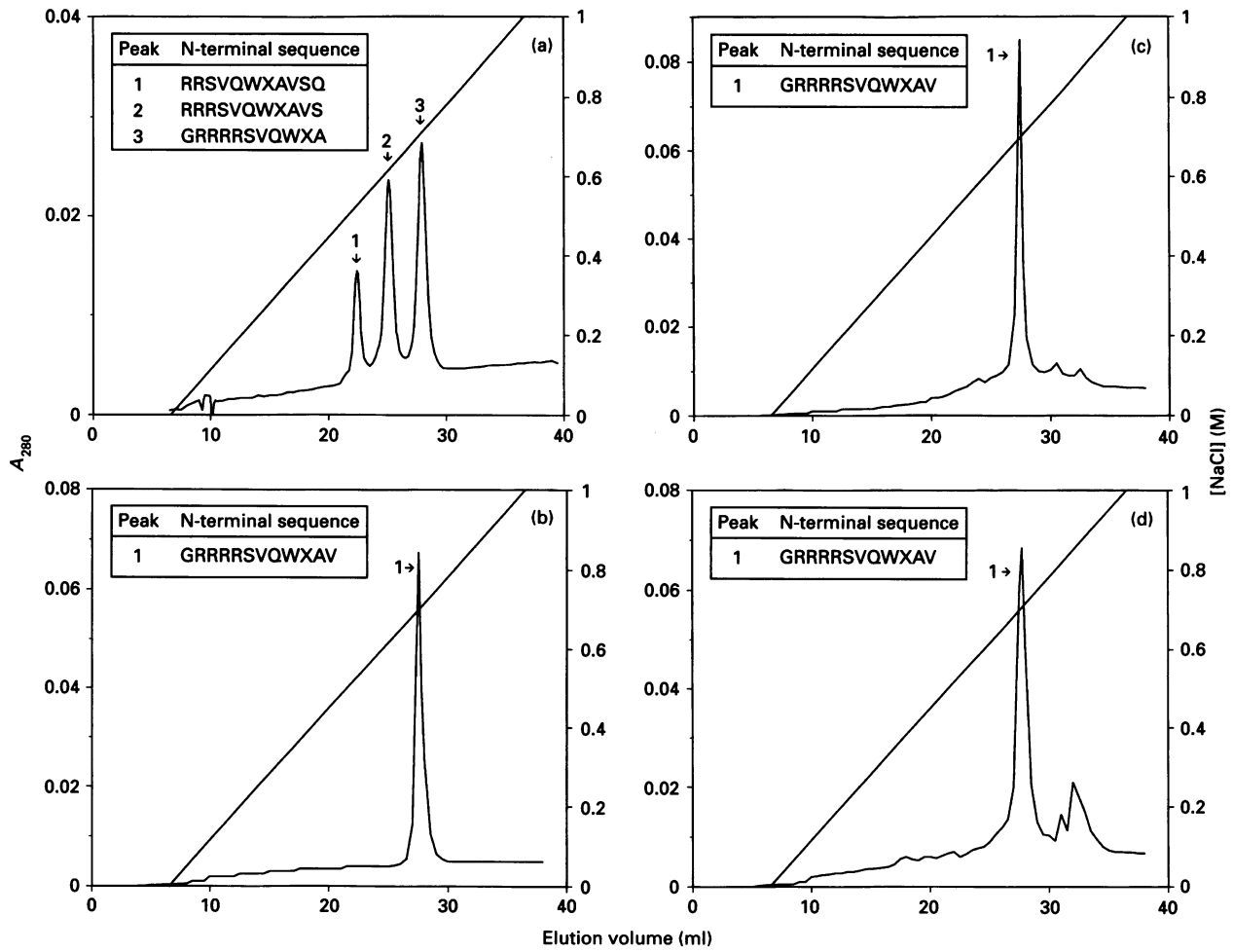

Figure 3 Mono S profiles of S Sepharose-purified natural hLF and rhLF

A 50 μg portion of purified human-milk-derived hLF from Sigma (**a**) or 100 μg samples of S Sepharose-purified hLF (**b**, natural hLF from fresh human milk; **c**, rhLF; **d**, rhLF, expressed in the presence of tunicamycin), were applied to a Mono S (HR 5/5; Pharmacia) cation-exchange column in 20 mM sodium phosphate, pH 7.5 (buffer A). Bound protein was eluted with a linear salt gradient of 0–1 M NaCl in 30 ml buffer A at a flow rate of 1.0 ml/min. Eluted protein was detected by absorbance measurement at 280 nm. The fractions applied to N-terminal protein sequencing with the automatic Edman degradation procedure (Applied Biosystems gasphase sequencer, model 473A) are indicated by numbers. Sequencing results are shown with the standard one-letter code for amino acids.

SDS/PAGE analysis of natural and rhLF

SDS/PAGE under non-reducing conditions of freshly diluted, non-boiled samples can be used to assess the degree of saturation with iron of hLF [20]. Figure 4 shows purified natural hLF (lane A) migrating as a doublet of protein bands designated the minor and major band at a ratio of about 1:9 respectively. The faster migration of both minor and major band of iron-saturated natural hLF (Figure 4, lane B) reflects the conformational change that occurred in hLF upon incorporation of iron [1]. The mobility of minor and major bands of non-boiled rhLF (lane D) is the same as that of non-boiled iron-saturated natural hLF (lane B). In addition, boiling of rhLF and iron-saturated natural hLF has the same effect on the mobility (compare lanes D and E with lanes B and C respectively). This indicates that rhLF is fully saturated with iron, with iron being released upon boiling. Absorption measurement indicated that the metal ion bound to rhLF was indeed iron; the A_{280}/A_{465} ratio for rhLF was identical with that of iron-saturated natural hLF, i.e. 24.0 [17]. Complete iron saturation of rhLF in culture medium is not surprising, as this medium contains 250 μM iron. The ratio of minor to major band in both non-boiled and boiled samples of rhLF (lanes D and E) is about 4:6.

The boiled sample of purified rhLF expressed in the presence of tunicamycin (lane G) shows a protein band with the same mobility as that in enzymically deglycosylated natural hLF (lane H). The migration of this protein is faster than that of major protein bands of boiled samples of natural hLF (lane C) and rhLF expressed without tunicamycin (lane E). This indicates that hLFNeo cells cultured with tunicamycin indeed express unglycosylated rhLF and that glycosylation heterogeneity accounts for the presence of a major and a minor band in natural hLF and in rhLF expressed without tunicamycin. The change in mobility of rhLF expressed with tunicamycin upon boiling (compare lanes F and G) indicates that unglycosylated rhLF is completely saturated with iron.

SDS/PAGE analysis of [^{35}S]rhLF

The results of N-terminal sequencing (Figure 3) and SDS/PAGE

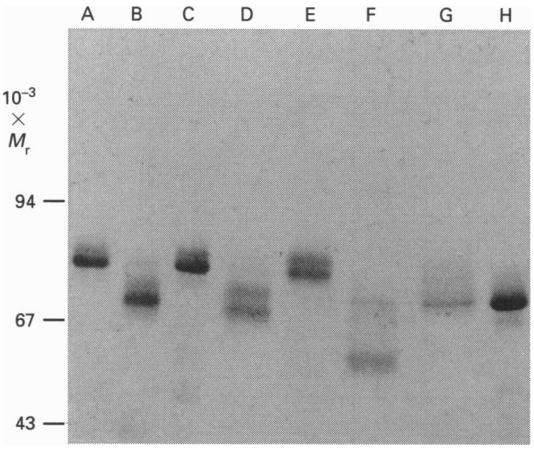

Figure 4 SDS/PAGE analysis of natural and rhLF

Fractions containing hLF that were eluted from Mono S at 0.7 M NaCl (Figure 3) were subjected to non-reducing SDS/7.5%-PAGE. Samples were diluted in SDS sample buffer and applied either directly or after boiling for 5 min. Lane A, boiled sample of natural hLF (5 μg); lanes B and C, non-boiled (B) and boiled (C) samples of iron-saturated natural hLF (5 μg); lanes D and E, non-boiled (D) and boiled (E) samples of purified rhLF (3 μg); lanes F and G, non-boiled (F) and boiled (G) samples of purified rhLF (2 μg) expressed in the presence of tunicamycin; lane H, boiled sample of enzymically deglycosylated natural hLF. Proteins were stained with Coomassie Brilliant Blue. Numbers on the left ($10^{-3} \times M_r$) indicate the migration of the protein standards.

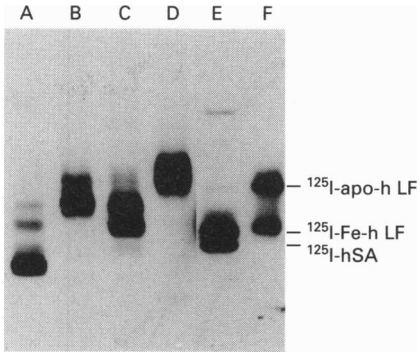

Figure 5 Autoradiography of SDS/PAGE analysis of metabolically labelled rhLF

[^{35}S]Methionine-labelled rhLF was immunoprecipitated with anti-hLF mAb 13.19 and subjected to non-reducing SDS/7.5%-PAGE. Lanes A and B, non-boiled (A) and boiled (B) immunoprecipitates of ^{35}S-labelled rhLF expressed in the presence of tunicamycin; lanes C and D, non-boiled (C) and boiled (D) immunoprecipitates of ^{35}S-labelled rhLF expressed without tunicamycin; lane E, non-boiled sample of iron-saturated hLF labelled with the ^{125}I-labelled Bolton–Hunter reagent [48] [lane E also contains ^{125}I-human serum albumin (hSA), because hSA was added as a carrier protein to the radioiodination mixture prior to gel filtration on a PD-10 column (Pharmacia)]; lane F, non-boiled sample of iron-saturated ^{125}I-hLF labelled by the chloramine-T method [49].

(Figure 4) of rhLF eluted at 0.7 M NaCl from Mono S do not give any evidence for proteolysis of rhLF during its synthesis and accumulation in tissue culture. This conclusion may, however, apply only to the protein eluted at 0.7 M NaCl from Mono S, since degradation products (such as hLF fragments lacking the strongly cationic N-terminal portion of intact hLF) may not have bound to Fast S at 0.4 M NaCl (see the Materials and methods section). Therefore we immunoprecipitated metabolically labelled rhLF with anti-hLF mAb 13.19, which recognizes an epitope in the C-lobe, from supernatant of hLFNeo cells. SDS/PAGE analysis of the immunoprecipitates (Figure 5), suggests that proteolysis of rhLF indeed does not occur during culture.

The results of Figure 5 furthermore confirm that both unglycosylated and glycosylated hLF can bind iron (Figure 4) and that saturation with iron occurs during culture, i.e. prior to purification.

Binding of natural and rhLF to hLZ and Re595 LPS

The binding of hLF to LPS has been implicated in its bactericidal and anti-inflammatory activity [5–7]. Also, the formation of complexes between hLF and human lysozyme (hLZ) may be important in the synergy between the antibacterial actions of these molecules [6,33]. Therefore we studied the role of hLF glycans in the interaction with hLZ and LPS.

Initial experiments in which two-fold serial dilutions of human lysozyme coupled to Sepharose were incubated with ^{125}I-hLF, revealed a dissociation constant (K_d, calculated as the lysozyme concentration at which half-maximal binding of hLF was observed) of 4.5×10^{-8} M for hLZ–hLF interaction. Next, we developed RIAs for hLF with hLZ–Sepharose and Re595 LPS–Sepharose to study the binding of distinct hLF species to these ligands. Figure 6 shows the dose–response curves of natural hLF and rhLF expressed with or without tunicamycin in the RIAs with hLZ–Sepharose (Figure 6a), with Re595 LPS–Sepharose (Figure 6b) and with mAb 13.17–Sepharose (Figure 6c). Correction of responses in RIAs with hLZ– and Re595 LPS–Sepharose for differences in hLF concentration with the RIA for hLF antigen (Figure 6c) revealed that the affinity of glycosylated and unglycosylated rhLF for hLZ and LPS did not differ from that of natural hLF. This indicates that the glycans of hLF are not involved in its binding to either hLZ or Re595 LPS.

Resistance of natural and rhLF to tryptic proteolysis

LF is known to be unusually resistant to degradation by the pancreatic proteolytic enzymes trypsin and chymotrypsin [34]. We studied the role of hLF glycan in the stability of hLF towards trypsin. Figure 7 shows the result of immunoblotting of SDS/PAGE-separated hLF immunoprecipitated from conditioned medium containing either natural hLF, glycosylated or unglycosylated rhLF to which trypsin had been added. Major cleavage products of natural hLF (lanes H and I) of M_r 51000 (designated C1) and M_r 39000 (designated N1) represent the C- and N-lobe respectively, as revealed by N-terminal protein sequencing (results not shown; see also [35]). Comparison of rhLF (lane F) and natural hLF (lane I) incubated with trypsin for 24 h suggests that rhLF is slightly more susceptible to tryptic proteolysis. This comparison also suggests that glycosylation heterogeneity in natural hLF as well as the difference in glycosylation between natural hLF and glycosylated rhLF resides in the C-lobe; a more prominent band of M_r 54000 (designated C2), probably representing the C-lobe with two N-glycan chains, is observed with glycosylated rhLF. The C1/C2 ratio in glycosylated rhLF is about 4:6 (lane F), whereas that in natural hLF is about 1:9 (lane I), i.e. these ratios are similar to those of minor to major bands in uncleaved molecules.

Unglycosylated hLF was almost completely digested in 4 h (lane B). The band of M_r 36000, designated NO, presumably represents the unglycosylated N-lobe. These results indicate that unglycosylated rhLF is much more susceptible to tryptic proteolysis than is glycosylated hLF.

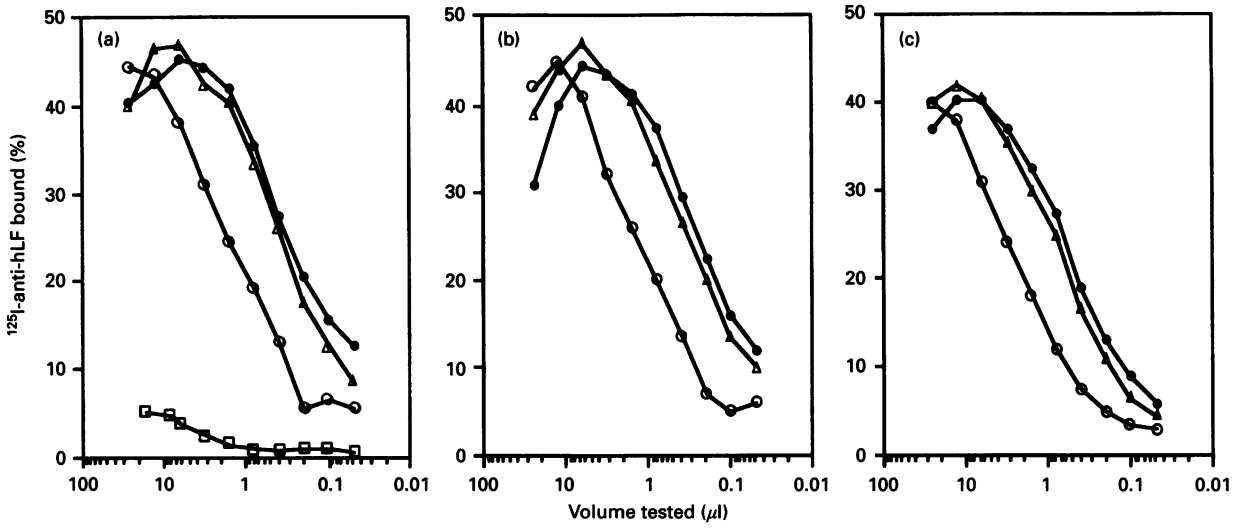

Figure 6 Binding of natural and rhLF to human lysozyme and Re595 LPS

Twofold serial dilutions of purified natural hLF (10 μg/ml, △), purified rhLF expressed with (○) or without (●) tunicamycin were tested in the RIAs with hLZ (**a**), Re595 LPS (**b**), and mAb 13.17 (**c**) coupled to Sepharose as described in the Materials and methods section. The specificity of hLF binding to hLZ, LPS and mAb 13.17 is demonstrated by the fact that no dose–response effect was observed when glycine-Sepharose (the amount of Sepharose was identical with that in hLZ–Sepharose) was incubated with serial dilutions of natural hLF (10 μg/ml, □, **a**).

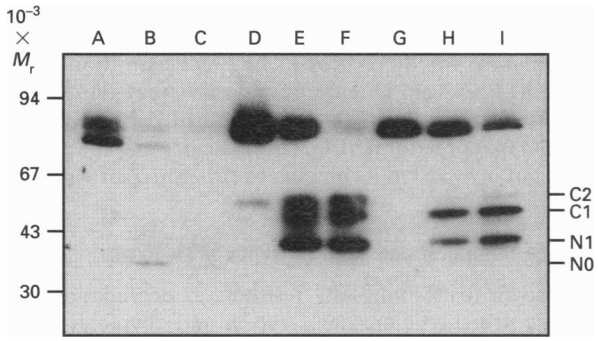

Figure 7 Susceptibility to tryptic proteolysis of natural and rhLF

SDS/PAGE and immunoblotting analysis of hLF immunoprecipitated from serum-free conditioned medium to which trypsin had been added; lanes A–C, rhLF expressed in the presence of tunicamycin that was incubated with trypsin for 0 h (A), 4 h (B), and 24 h (C); lanes D–F, rhLF expressed without tunicamycin that was incubated with trypsin for 0 h (D), 4 h (E), and 24 h (F); lanes G–I, natural hLF that was incubated with trypsin for 0 h (G), 4 h (H, and 24 h (I). C2, C1, N1 and N0 are explained in the text.

DISCUSSION

In the present paper we show that lack of glycosylation of hLF does not affect several properties of this protein that may be relevant to its anti-infective and anti-inflammatory action *in vivo*.

Recombinant hLF has been expressed in a non-human cell line [36], in fungi [37], yeast [38] and transgenic mice [39]. To our knowledge there is no systematic study of the possible functional consequences of differential glycosylation in natural or recombinant hLF. In addition, all published studies concerning biological relevance of glycosylation of natural hLF employed enzymically (partially or fully) deglycosylated protein. A major drawback of that approach is that the possibility cannot be ruled out that a distinct biological activity of an enzymically deglycosylated protein does not relate to the loss of sugar residues or glycan chains, but is the result of processes intrinsic to the experimental protocols employed. For example, in most cases, denaturing agents are used to accomplish complete enzymic deglycosylation [21].

To circumvent such problems we have chosen to express recombinant hLF in its glycosylated and (completely) unglycosylated form in a human cell line. Analysis of expression by immunoassay revealed that rhLF is immunologically identical with natural hLF (Figure 2). The presence of intact unglycosylated rhLF in the supernatant of hLFNeo cells cultured with tunicamycin (Figures 2, 3d and 4), may indicate that N-glycosylation of hLF is no absolute requirement for its secretion. However, it cannot as yet be excluded that unglycosylated rhLF present in culture medium has not been secreted by living cells, but was released upon lysis of cells.

Mono S chromatography and N-terminal sequencing (Figure 3) revealed equal cationic properties and intact N-terminal sequences of natural hLF, glycosylated and unglycosylated rhLF. We have not been able to reproduce the observation of Makino and Nishimura that apo- and iron-saturated lactoferrin are eluted at different positions from a Mono S column [40]. Several purified hLF preparations from commercial suppliers appeared to be degraded at their N-termini: hLF eluting from a Mono S column at 0.5 and 0.6 M NaCl appeared to represent hLF lacking three and two N-terminal residues respectively, whereas the native protein is eluted at 0.7 M NaCl (Figure 3a). Similar N-terminal degradation has previously been observed in hLF of maternal origin in the urine of preterm infants [35] and in rhLF expressed by *Aspergillus oryzae* [37]. We now use Mono S chromatography as a simple means to assess the N-terminal integrity of hLF preparations, because the N-terminus of hLF plays a decisive role in its biological action. For example, the N-terminus has been implicated in its clearance from the circulation [41], in binding to glycosaminoglycans [42] and LPS [7], and in its bactericidal effect [5].

Recombinant hLF expressed in the presence of tunicamycin

showed the same increased mobility on SDS/PAGE as enzymically deglycosylated natural hLF, i.e. it appeared indeed unglycosylated (Figures 4 and 5). Results of SDS/PAGE (Figures 4 and 5) and N-terminal sequencing (Figure 3) of native and enzymically deglycosylated natural hLF, as well as of rhLF, provide definite proof that the major and minor hLF bands on SDS/PAGE indeed result from glycosylation heterogeneity [21] and not from incomplete removal of the hLF signal sequence, as has been suggested [20]. We observed that glycosylation of rhLF was different from that in natural hLF (Figure 4). This difference may be explained by the fact that rhLF was expressed by a human kidney-derived cell line, whereas natural hLF was expressed by human mammary-gland epithelium. For example, Spik et al. have reported that the carbohydrate sequence and composition of lactoferrins vary between animal species [19,43,44], its site of production [18,19] and even at one production site [19,44].

Tryptic proteolysis of hLF species (Figure 7) suggests that N-glycosylation heterogeneity in natural and rhLF resides in the C-lobe of hLF. We speculate that the optional N-glycosylation site (asparagine 624; not used according to [18]) in hLF is actually used in about 10 and 40% of natural and rhLF molecules respectively. Such molecules would account for the minor bands on SDS/PAGE and bear three glycan chains, whereas major bands would represent molecules bearing two glycan chains.

Both glycosylated and unglycosylated rhLF were completely saturated with iron according to SDS/PAGE analysis, i.e. they were able to bind iron and to retain it even in the presence of SDS. This indicates that the N-linked glycans of hLF may not be essential in maintaining the stability of the iron-saturated conformation. Legrand et al. have reported that enzymic deglycosylation of tryptic hLF N-lobe fragments of M_r 20000 and 30000 abrogated or reduced the iron-binding capacity by 2-fold respectively. These investigators have suggested that interactions between the glycan moiety and protein backbone are important for stabilization of the iron-binding site [24]. However, no data are available on iron-binding and release of enzymically deglycosylated intact (non-cleaved) hLF polypeptide. Such data may indeed be relevant, since Day et al. have recently stated that interaction of the C-lobe with the N-lobe plays a crucial role in the stabilization of the iron-saturated conformation [45].

Since even unglycosylated rhLF was able to bind iron, we expect that natural hLF and glycosylated rhLF will behave identically or very similarly with respect to those biological effects based on iron binding. Apart from scavenging free iron, direct binding of hLF to LPS of Gram-negative bacteria with disturbance of cell-wall stability has been implicated in hLF antibacterial action [5–7]. The binding of hLF to the lipid A moiety of LPS may account for the reduced production of cytokines upon challenge with LPS [9,10]. The identical affinities of natural hLF, glycosylated and unglycosylated rhLF for LPS (Figure 6b) provides support for the notion that hLF glycans are not involved in binding to LPS. In addition, the identical affinities (K_d 4.5×10^{-8} M) of natural hLF, glycosylated and unglycosylated rhLF for hLZ (Figure 6a) indicates that hLF glycan apparently is not involved in the interaction of hLF with hLZ. We speculate that direct intermolecular interaction is one of the possible mechanisms to explain for the previously described synergy between antibacterial action of hLF and hLZ [6]. Human LF may act as a vehicle to expose inner-cell-wall components of Gram-negative bacteria to hLZ enzymic activity.

Unglycosylated rhLF appeared much more susceptible to tryptic proteolysis than its glycosylated counterpart (Figure 7). It has recently been discovered that enzymic hydrolysates of bovine LF have bactericidal properties more potent than native bovine LF [5]. However, the physiological consequence of increased susceptibility for proteolysis of unglycosylated rhLF cannot be predicted, since the mechanism of hLF action *in vivo* is as yet incompletely understood.

In conclusion, our results provide no evidence for an important contribution of hLF glycan chains to activities related to iron binding and interaction with LPS and hLZ.

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