Biosynthesis and Processing of Lactoferrin in Bone Marrow Cells,  
A Comparison With Processing of Myeloperoxidase

By Inge Olsson, Mikael Lantz, Ann-Maj Persson, and Kristina Arnjots

The processing and intracellular transport of lactoferrin of the neutrophil specific granules was investigated by biosynthetic labeling with \((^{14}C)\)leucine of bone marrow cells from healthy individuals and patients with chronic myeloid leukemia. Lactoferrin was precipitated with antilactoferrin serum and the immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS), polyacrylamide gel electrophoresis (PAGE) followed by fluorography. In contrast to myeloperoxidase of azurophil granules, lactoferrin was not synthesized as a larger precursor, and it was not found to be phosphorylated. The transfer to granules of newly synthesized lactoferrin was demonstrated in pulse–chase labeling experiments followed by centrifugation of cell homogenate in a Percoll gradient. Monensin, which exchanges protons for Na\(^+\) and NH\(_4\)^{+} cation, blocked the transfer completely, indicating a need for acidification mechanisms. Unlike myeloperoxidase, newly synthesized lactoferrin rapidly became resistant to endoglycosidase H, indicating a transport through the medial and transister-nae of the Golgi apparatus with conversion of “high man- nose” to “complex” oligosaccharide side chains. Intracellular transfer of some major neutrophil azurophil and specific granule constituents is obviously regulated differently. Lactoferrin seems to be processed like proteins destined for secretion, while myeloperoxidase is processed more or less like lysosomal enzymes.

© 1988 by Grune & Stratton, Inc.

---

EXPERIMENTAL

Chemicals. Sodium \(^{32}P\)-phosphate (carrier free), \((^{2}H)\)mannose (27.2 Ci/mmol), \((^{14}C)\)leucine (342 mCi/mmol), \(^{3}H\)-uridine (7.1 Ci/mmol), and En3Hance were obtained from New England Nuclear (Dréieich, Germany). Protein A-Sepharose CL-4B and Perkoll were from Pharmacia (Uppsala, Sweden). Endo-N-acetyl-glucosaminidase H (endoglycosidase H) was from Miles Laboratories Inc (Rehovot, Israel). Phenylmethanesulphonyl fluoride, Chloroquine, and monensin were from Sigma Chemical Co (St Louis). Acrylamide/bisacrylamide (29:1) was from Bio-Rad (Richmond, CA).

Isolation of bone marrow cells. After informed consent had been obtained, bone marrow cells from healthy individuals and from patients with chronic myeloid leukemia were collected in heparinized McCoy's medium and separated on Isopaque/Ficoll (1.077 g/mL). The cells from the interphase, which consist of immature myeloid cells devoid of mature granulocytes, were washed and used for biosynthetic labeling.

Labeling of cells. Bone marrow cells were incubated in leucine-free minimum essential medium (Eagle) with 1% fetal bovine serum (FBS) at 37° for 30 hours to allow the depletion of the intracellular leucine pool. The labeling medium was also leucine free but contained 10% FBS, which resulted in optimal biosynthetic labeling of lactoferrin and myeloperoxidase (data not shown). The cells, 2 x 10^6/mL, were incubated with 10 \(\mu\)Ci of \((^{14}C)\)leucine/mL. In chase experiments cells were pulsed for 15 to 60 minutes with 30 \(\mu\)Ci of \((^{14}C)\)leucine/mL, washed with and resuspended in RPMI 1640 medium with 10% FBS at a density of 10^6 cells/mL, and incubated for 20 hours (chase). In phosphorylation experiments cells were incubated in phosphate-free minimum essential medium (Eagle) supplemented with 10% FBS and carrier-free inorganic \((^{3}H)\)phosphate, 250 \(\mu\)Ci/mL.

Labeling of cells with \((^{2}H)\)mannose was performed in glucose-free RPMI 1640 supplemented with 0.5 mmol/L glucose, 10 \(\mu\)g of insulin/mL, and 10% FBS. The cells, 2 x 10^6/mL, were incubated with 100 \(\mu\)Ci of \((^{2}H)\)-mannose/mL for 20 hours.

Subcellular fractionation. Homogenization was done at a concentration of 10^6 cells/mL in 0.34 M-sucrose/5 mmol/L HEPES from the Division of Hematology, Department of Medicine, University of Lund, Sweden.

Submitted November 28, 1986; accepted October 6, 1987.
Supported by the Swedish Cancer Society, Alfred Österlund Foundation, and the Medical Faculty of Lund.
Address reprint requests to Inge Olsson, MD, Research Department 2, E-blocket, Lund Hospital, S-221 85 Lund, Sweden.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1988 by Grune & Stratton, Inc.
0006-4971/88/7102-0023$3.00/0
(pH 7.3)/0.5 mmol/L EDTA with 40 strokes of a Dounce glass homogenizer. The homogenate was diluted with the same solution, and unbroken cells and nuclei were pelleted by centrifugation at 700 g for ten minutes. For density gradient separations, 6 mL of 30% Percoll in HEPES/sucrose adjusted to give a final concentration of 15 mmol/L HEPES pH 7.4 and 0.25 mol/L sucrose, were layered on top of a 1-mL cushion of saturated sucrose. The 700 g supernatant of the cell homogenate (2 mL) was layered on top of the Percoll. Centrifugation was carried out at 32,000 g for 60 minutes in a Sorvall RC-5B centrifuge using the SE-12 rotor. Fractions were collected with a peristaltic pump. In studies of the subcellular processing of lactoferrin and myeloperoxidase, the fraction volume was 0.8 mL, except for the last fraction (no. 10) in which all the cytosol was collected. Refractory indices were determined by use of a refractometer to calculate the density.

Immunochromatography by single radial immunodiffusion of the total myeloperoxidase and lactoferrin content of the fractions was used for localization of the azurophil and specific granules, respectively. Galactosyl transferase activity was determined as a marker for the distribution of Golgi elements in the gradient. β-hexosaminidase activity was determined as previously described and was used as a marker for lysosomes. The distribution in the gradient of plasma membrane was determined by measuring the radioactivity of the fractions after prior labeling of the cell surface with [3H]leucine.

For identification of subcellular sites for newly synthesized protein, cells (10 mL, 3 x 10⁶/mL) were incubated at 37°C for ten minutes with 50 μCi of [14C]leucine/ml, followed by the addition of cycloheximide, 100 μg/mL, which was also added to the homogenization medium. Ribosomal RNA was identified by incubation at 37°C for two hours of 8 mL of cells (10⁶/mL) with 10 μCi of [3H]uridine/mL of RPMI 1640 medium with 10% FBS. Homogenates of cells labeled with [14C]leucine or [3H]uridine were subjected to Percoll density gradient centrifugation. The distribution of macromolecular incorporation of radioisotope-labeled precursor was determined by trichloroacetic acid precipitation of aliquots from the Percoll fractions followed by counting of radioactivity.

Immunoprecipitation of radioactive lactoferrin and myeloperoxidase. Extraction was done with a radioimmunoprecipitation assay (RIPA) buffer, which consisted of 0.15 mol/L NaCl/30 mmol/L HEPES (pH 7.3)/1% (vol/vol) Triton X-100/1% (wt/vol) sodium deoxycholate/0.1% (wt/vol) sodium dodecyl sulphate (SDS). Approximately 1 mL of RIPA buffer containing 1 mmol/L phenylmethanesulphonyl fluoride was used per 10 cells. The extracts were kept on ice for one hour and cleared by centrifugation at 4°C for 30,000 g for two hours. The resulting supernatant was stored frozen until it was used for immunoprecipitation. Percoll-density-gradient fractions were diluted with one half volume of a fivefold concentrated RIPA buffer and with 3 vol of H2O. The fractions were then clarified by centrifugation at 4°C at 32,000 g for two hours, and the supernatants were stored at 4°C until used for immunoprecipitation.

RIPA buffer extracts, 100 to 300 μL, of whole cells were mixed with 15 μL of anti lactoferrin or anti myeloperoxidase produced by immunization of rabbits. After being left on ice overnight, 40 μL of a Protein A-Sepharose (200 mg/mL) solution in RIPA buffer was added for collection of the immunoprecipitate by rotation at 4°C for 30 to 50 minutes. For immunoprecipitation of the RIPA buffer extract of Percoll-density-gradient fractions, the appropriate amount of specific antiserum was bound to the Protein A-Sepharose before addition of the extract. Thereafter the mixture was rotated overnight. All Protein A-Sepharose samples were washed five times with RIPA buffer, and the supernatant was carefully removed. The pellet was resuspended in 50 μL of water plus 15 μL of electrophoresis sample buffer (0.4 mol/L Tris/HCl[pH 6.8]/50% [vol/vol] glycerol/10% [wt/vol] SDS/5% [wt/vol] mercaptoethanol), boiled for five minutes, and electrophoresed.

SDS/polyacrylamide gel electrophoresis and fluorography. SDS/polyacrylamide gel electrophoresis (PAGE) was carried out on slab gels 18-cm long, 1.5-mm thick, and 16-cm wide, with ten wells, in an LKB 2001 vertical electrophoresis unit (LKB Products, Bromma, Sweden) according to Laemmli. Samples were applied on a linear gradient of 5% to 20% polyacrylamide gel with a 3% stacking gel. The electrophoresis was run at 25 mA/gel without cooling. Gels were fixed in 10% (wt/vol) trichloroacetic acid/10% acetic acid/30% (vol/vol) methanol for at least one hour and treated with EnHance for one hour and water for one hour. They were dried on filter paper and exposed to x-ray film (Kodak X-Omat S) at −80°C for two to four days.

Apparent molecular weight values were determined by use of the following (14C)-methylated standards (New England Nuclear, Boston): Cytochrome c (mol wt 12,300), carbonic anhydrase (mol wt 30,000), ovalbumin (mol wt 46,000), bovine serum albumin (mol wt 69,000), and phosphorylase b (mol wt 97,400).

Results

Bone marrow cells from both healthy individuals and patients with chronic myeloid leukemia were used in this study. Identical results were obtained in both cases for biosynthetic labeling and processing of lactoferrin and myeloperoxidase (data not shown). Therefore the results presented are based on experiments with marrow cells from patients with chronic myeloid leukemia because such cells were easily available in large numbers. Also, cells from the peripheral blood of patients with chronic myeloid leukemia were found suitable for biosynthetic labeling of lactoferrin. This is in agreement with a previous report that showed abundant active lactoferrin biosynthesis in normal bone marrow and in both the bone marrow and peripheral blood of patients with chronic myeloid leukemia if the samples contained substantial numbers of myelocytes and metamyelocytes.
BIOSYNTHESIS OF LACTOFERRIN 443

Subcellular transport of newly synthesized lactoferrin. A self-generating Percoll density gradient was used for separation of subcellular organelles (Fig 1). When the post-nuclear supernatant was separated on Percoll, several bands were visible. Markers for azurophil granules, eg, myeloperoxidase and β-hexosaminidase, were concentrated to the bottom of the gradient, indicating the localization of azurophil granules. Both myeloperoxidase and lactoferrin showed a similar distribution, indicating that there was no clear separation between azurophil and specific granules. Golgi-derived elements identified by the distribution of galactosyl transferase, plasma membranes identified by the distribution of surface-bound 125I-lectin, and subcellular sites for protein synthesis as well as ribosomal RNA were found in the upper part of the gradient. No clear separation was achieved between the latter organelles, but a clear separation was achieved between granules and other organelles. Pretreatment of the cells with 1 μmol/L Monensin or 10 mmol/L NH₄Cl for 20 hours resulted in a slight change of the distribution in the gradient of a minor fraction of the myeloperoxidase and lactoferrin (Fig 1D). Thus, in particular with Monensin, a slightly increased amount of these proteins was recovered at the top of the gradient. However, the distribution in the gradient of the bulk of the granule proteins was unchanged.

After 30 minutes of incubation with 14Cleucine (Fig 2), radiolabeled lactoferrin was widely distributed in the gradient except in the most dense fractions corresponding to granules. However, it was not only present in light-density organelles corresponding to endoplasmic reticulum and Golgi but also in fractions with an intermediate density. The latter may represent pregranule structures because their density is higher than that for Golgi elements. In addition to the mol wt 79,000 component corresponding to native lactoferrin, labeled species with mol wt of approximately 60,000 and 40,000 to 45,000 were visible. These are regarded as degradation products because they were not present after very short incubation times (Fig 5). During a 20-hour chase of the label, most of the newly synthesized lactoferrin accumulated in the granule-containing-high-density fractions, although some remained in the cytosol and in fractions with an intermediate density.

Effects of monensin and NH₄Cl on subcellular transport of lactoferrin and myeloperoxidase. Figure 3 shows subcellular distribution in the Percoll gradient of newly synthesized lactoferrin. Cells were pulse labeled with 14Cleucine for 30 minutes, after which the label was chased for 20 hours (1) without addition, (2) with addition of 1 μmol/L monensin, or (3) with addition of 10 mmol/L NH₄Cl. Both agents inhibited the transfer of lactoferrin to organelles with a high density, and labeled protein remained in organelles corresponding to Golgi and endoplasmic reticulum. These results undoubtedly indicate a block in transfer because, as mentioned above, monensin and NH₄Cl did not change the distribution in the gradient of the bulk of granule proteins. The ionophore monensin catalyzes the exchange of monovalent cations across membranes, and it has been suggested that monensin may disrupt a gradient that is important for budding of vesicles from the Golgi.36 NH₄Cl interferes with the normal acidification of the vacuolar system of the cell and of lysosomes. Our data indicate that monensin and NH₄Cl either block the transport of lactoferrin to specific granules or block the granule formation itself.

We18 and others37 have demonstrated that 10 mmol/L NH₄Cl does not interfere with the processing and subcellular transport of newly synthesized myeloperoxidase in the leukemic promyelocytic cell line HL-60. Because this result pointed to different effects on azurophil (myeloperoxidase)
and specific (lactoferrin) granule constituents, the experiment was repeated by use of bone marrow cells (Fig 4). After one-hour incubation with \(^{14}C\)leucine, radiolabeled myeloperoxidase was present as the mol wt 90,000 and mol wt 82,000 precursor polypeptides located in organelles corresponding to endoplasmic reticulum and Golgi elements. During a 20-hour chase of the label, maturation of MPO occurred into polypeptides with mol wt values of 62,000 and 12,000. Mature myeloperoxidase was located in granule fractions but also in fractions with lower density, indicating that processing occurred also in pregranular structures. It was reported previously for HL-60 cells\(^{16}\) that NH\(_4\)Cl did not interfere profoundly with processing. The present results for bone marrow cells show that maturation of myeloperoxidase takes place also in the presence of NH\(_4\)Cl, but inhibition of transfer to granules or, alternatively, inhibition of granule formation was indicated.

Lack of phosphorylation of lactoferrin. Incubation of marrow cells with \(^{32}P\)phosphate did not result in detectable phosphorylation of lactoferrin, as judged by results from separate experiments performed on cells from three individuals (data not shown). This is in contrast to myeloperoxidase, which is phosphorylated by incubation with \(^{32}P\)phosphate.\(^{16}\)

Acquisition of endo H resistance. Newly synthesized lactoferrin rapidly became resistant to digestion with endo H (Fig 5). Thus already after pulse labeling for 25 minutes approximately 50% of the polypeptide was resistant, indicating conversion of a “high mannose” to a “complex” sugar configuration of N-linked oligosaccharides, which occurs in the medial and transcisternae of the Golgi apparatus.\(^{31,32}\) Subsequent chase of labeled lactoferrin demonstrated virtually complete resistance to endo H in less than 15 minutes. This result is in contrast to results for myeloperoxidase where both the precursor forms and the large mature subunit remained susceptible to endo H (Fig 5).

DISCUSSION
The lactoferrin production during myeloid cell differentiation is highly regulated and is a genetic marker for terminal neutrophil maturation.\(^{33}\) Lactoferrin has been localized to specific (secondary) granules of neutrophils\(^{14}\) produced in myelocytes.\(^{2}\) Therefore biosynthesis of myeloid granule protein biosynthesis must be investigated in cell systems containing immature cells with active granulogenesis. In a previous study\(^{29}\) as well as in the present study it was demonstrated that normal or chronic myeloid leukemia

---

**Fig 2.** Pulse-chase labeling with \(^{14}C\)leucine of lactoferrin and distribution of labeled lactoferrin in subcellular fractions. Cells were pulse labeled with \(^{14}C\)leucine for 30 minutes, and the label was chased for 20 hours. The postnuclear supernatants obtained after pulse (a) and 20-hour chase (b) were centrifuged on 30% Percoll for separation of subcellular organelles, and fractions were collected and analyzed. Extraction, immunoprecipitation with anti-lactoferrin serum, SDS-PAGE, and fluorography were performed as described in the text. The positions of the immunoprecipitated lactoferrin, UF (mol wt 79,000) is indicated to the right; mol wt markers are shown to the left. The relative distribution of markers for subcellular organelles is as in Fig 1.
BIOSYNTHESIS OF LACTOFERRIN

Fig 3. The effect of monensin and NH₄Cl on the processing of lactoferrin during chase of [¹⁴C]leucine-labeled lactoferrin. Cells were pulse labeled with [¹⁴C]leucine for 30 minutes, and the label was chased for 20 hours without addition (a), in the presence of 1 µmol/L monensin (b), and in the presence of 10 mmol/L NH₄Cl (c). The postnuclear supernatants obtained after chase were centrifuged on 30% Percoll. The densities of the fractions obtained were as shown in Fig 1, and the position of immunoprecipitated lactoferrin (LF) is indicated. Extraction, immunoprecipitation with anti-lactoferrin serum, SDS-PAGE, and fluorography were performed as described in the text. The relative distribution of markers for subcellular organelles is as in Fig 1.

Fig 4. Pulse-chase labeling with [¹⁴C]leucine of myeloperoxidase and distribution of labeled myeloperoxidase in subcellular fractions. Cells were pulse labeled with [¹⁴C]leucine for 30 minutes, and the label was chased for 20 hours. The postnuclear supernatants obtained after pulse (a) and 20-hour chase without (b) and with (c) 10 mmol/L NH₄Cl were centrifuged on 30% Percoll for separation of subcellular organelles, and fractions were collected and analyzed. Extraction, immunoprecipitation with antimyeloperoxidase serum, SDS-PAGE, and fluorography were performed as described in text. The position of the myeloperoxidase precursor polypeptides, mol wt 90,000 (90) and mol wt 82,000 (82), as well as the two mature myeloperoxidase subunits, mol wt 62,000 (62) and mol wt 12,000 (12) is indicated to the right; mol wt markers are shown to the left. This experiment was performed with the same cells as in Fig 3.

Marrow cells can be used to investigate the biosynthesis of a specific granule protein such as lactoferrin.

A considerable interest is documented in the synthesis and traffic control of lysosomal enzymes. These require phosphorylation of mannose residues of oligosaccharide side chains. Phosphorylation takes place in the cis part of the Golgi apparatus followed by attachment to a receptor for mannose-6-phosphate, which directs transfer to lysosomes. Concomitant with this transfer, lysosomal enzymes also undergo proteolytic cleavage of the proenzyme, resulting in formation of mature enzyme. Azurophil granules are lysosomal-like. Therefore it has been important to investigate if one
major constituent, myeloperoxidase, is processed like typical lysosomal enzymes. Data have shown that myeloperoxidase is synthesized as a larger precursor,\textsuperscript{15-16} which is modified by proteolytic processing in the pregranular compartment.\textsuperscript{14,16} Phosphorylation of oligosaccharide side chains of myeloperoxidase takes place, but it is uncertain if it is of importance for directing intracellular transport.\textsuperscript{16} Lactoferrin-containing specific granules do not contain lysosomal enzymes.\textsuperscript{5,6} According to the present results there are differences in processing of myeloperoxidase and lactoferrin, indicating that constituents of azurophil and specific granules are processed differently. Thus unlike myeloperoxidase, lactoferrin did not seem to be synthesized as a larger precursor, and it was not found to be phosphorylated.

To investigate the intracellular transport of lactoferrin, Percoll density gradient centrifugation was adopted for separation of subcellular organelles. A clear separation was achieved between cytoplasmic granules and organelles of lower density. Thus the high-density fractions were completely devoid of biochemical markers for Golgi elements, endoplasmic reticulum, or plasma membrane. Although the low density part of the gradient contained several visible bands, the fractions collected did not show clear separation of Golgi elements, endoplasmic reticulum, and plasma membranes, as judged by the distribution of the biochemical markers. Anyhow, our data demonstrated that newly synthesized lactoferrin was transferred with time to the high-density fractions where specific granules reside. However, some newly synthesized lactoferrin remained in lower density organelles also after 20 hours of chase, indicating contamination with granule material, release of granular lactoferrin during centrifugation, or inefficient granule formation.

The observed complete monensin-induced interruption of intracellular progression of newly synthesized lactoferrin may be explained by disruption of the transfer through the Golgi apparatus or by inhibition of granule formation itself. The finding that NH\textsubscript{4}\textsuperscript{+} had an identical effect indicates that acidification mechanisms are important for the transfer of specific granule proteins to proceed normally. This is in contrast to findings for myeloperoxidase, the processing of which was not interrupted by NH\textsubscript{4}\textsuperscript{+}, but the intracellular transport was partly inhibited or slower than normal.

Our results demonstrate that newly synthesized lactoferrin resistant to endo H was visible within 30 minutes of biosynthesis. This shows that lactoferrin is transported through the medial and transisternae of the Golgi apparatus, where conversion to “complex” oligosaccharides resis-

Fig 5. Endo H digestion of lactoferrin and myeloperoxidase. Bone marrow cells were pulse labeled for 20 minutes and then chased for time periods as indicated in the figure. After immunoprecipitation with antimeyeloperoxidase or anti-lactoferrin serum, each sample was digested in the presence or absence of endo H, followed by electrophoresis and fluorography as described in text. The positions of the myeloperoxidase precursor polypeptides, mol wt 90,000 and mol wt 82,000, as well as the two mature myeloperoxidase subunits, mol wt 62,000 and mol wt 12,000, are indicated. Also indicated is the position of lactoferrin, mol wt 79,000. Myeloperoxidase, MPO; lactoferrin, LF.
tant to Endo H occurs. In contrast, both precursor and mature myeloperoxidase subunits remained susceptible to endo H, which may indicate that myeloperoxidase is not transported through the medial and trans part of the Golgi apparatus at all. Myeloperoxidase-containing vesicles, which form azurophil granules, may be released directly from the cis part of Golgi. Results from one report indicated that the cis Golgi cisternae represent the sites where the secretory and lysosomal pathways diverge; lysosomal enzymes bearing the mannose-6-phosphate recognition marker bind to mannose-phosphate receptors in this location and are delivered to lysosomes via coated vesicles.

In conclusion, this work has demonstrated that the processing and intracellular transfer of a major neutrophil-specific granule constituent, lactoferrin, is regulated differently than that for a major azurophil granule constituent, myeloperoxidase. Lactoferrin seems to be processed like proteins destined for secretion, while myeloperoxidase is processed more like lysosomal enzymes.

REFERENCES

Biosynthesis and processing of lactoferrin in bone marrow cells, a comparison with processing of myeloperoxidase

I Olsson, M Lantz, AM Persson and K Arnljots