Lactoferrin-deficient neutrophil polymorphonuclear leucocytes in leukaemias: a semiquantitative and ultrastructural cytochemical study

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SUMMARY Semiquantitative analysis of lactoferrin deficiency in neutrophil polymorphonuclear leucocytes in various haematological and non-haematological disease was carried out by scoring polymorphonuclear leucocytes stained for lactoferrin by the immunoperoxidase method. The staining patterns for lactoferrin were classified into four types (0-III) based on the intensity of reaction, and the sum of the ratings of 100 polymorphonuclear leucocytes was considered as "lactoferrin score" with a possible range of 0-300. As a result, significantly low lactoferrin-scores were frequently observed in acute leukaemias and the acute phase of chronic leukaemias. Of 35 cases with leukaemias, lactoferrin-negative polymorphonuclear leucocytes (type 0) were observed in the following cases: eight cases of acute myelogenous leukaemia (8/14), a case of chronic myelogenous leukaemia (1/10) in blast crisis, one of acute promyelocytic leukaemia (1/1), one of acute monocytic leukaemia (1/2), and a case of chronic myelomonocytic leukaemia (1/2) in a transitional phase to an acute myelomonocytic leukaemia. In two cases of acute myelogenous leukaemia, in which the majority of polymorphonuclear leucocytes were negative for lactoferrin, ultrastructural cytochemical study revealed total lack of specific granules in these polymorphonuclear leucocytes. This suggests that lactoferrin is localised in the specific granules of neutrophils as has been postulated previously by others.

Lactoferrin is a protective agent against microbial organisms^{1,2} found in many body fluids¹ and exocrine cells^{1,3} of various species. Masson *et al*⁴ discovered that lactoferrin is also present in human neutrophil polymorphonuclear leucocytes, and later fractionation studies revealed that lactoferrin is localised in specific granules of rabbit heterophils⁵ and human neutrophils.⁶ By means of a light microscopic immunocytochemical method, Mason⁷ and Pryzwansky *et al*⁸ demonstrated lactoferrin in late myeloid precursors. These morphological studies also indicated the presence of lactoferrin in specific granules of neutrophils. Like the enzyme cytochemical staining for myeloperoxidase, which has been used as a marker for primary granules, the immunocytochemical demonstration of lactoferrin has recently been applied for clinical medicine as a marker for neutrophil specific granules.⁹ In cer-

tain leukaemias^{10,11} and congenital neutrophil anomalies,¹²⁻¹⁴ the presence of lactoferrin-deficient polymorphonuclear leucocytes has been reported by some workers.

In the present study, in order to find the clinical and pathological significance of lactoferrin deficiency in neutrophils, semiquantitative analysis of lactoferrin deficiency in polymorphonuclear leucocytes was carried out by means of a scoring technique. The scores for lactoferrin were compared among various haematological and non-haematological disorders. This study also describes the ultrastructure of lactoferrin-deficient polymorphonuclear leucocytes observed in two cases of acute myelogenous leukaemia.

Material and methods

PATIENTS STUDIED

Peripheral blood or bone marrow smears were

Table 1 Summary of cases with lactoferrin-negative polymorphonuclear leucocytes (type 0)

	No of cases
Leukaemias	
Acute myelogenous (AML)	8/14
Chronic myelogenous (CML)	0/8
Blast crisis of CML	1/2
Acute promyelocytic (APL)	1/1
Acute monocytic (AMoL)	1/2
Acute myelomonocytic (AMMoL)	0/1
Chronic myelomonocytic (CMMoL)	1/2
Acute lymphoblastic (ALL)	0/2
Chronic lymphocytic (CLL)	0/2
Hairy cell leukaemia	0/1
Other haematological diseases	
Idiopathic thrombocytopenic purpura	0/9
Aplastic anaemia	0/3
Polycythaemia vera	0/2
Primary myelofibrosis	0/2
Malignant lymphoma	0/2
Multiple myeloma	0/1
Hereditary spherocytosis	1/1
Histiocytosis-X	0/1
Non-haematological diseases	2/24
Total	15/80

obtained from 80 patients with various haematological or non-haematological diseases seen in the Keio University Hospital. These included 14 cases of acute myelogenous leukaemia (AML), 10 of chronic myelogenous leukaemia (CML), one of acute promyelocytic leukaemia (APL), two of acute monocytic leukaemia (AMoL), one of acute myelomonocytic leukaemia (AMMoL), two of chronic myelomonocytic leukaemia (CMMoL), two of acute lymphoblastic leukaemia (ALL), two of chronic lymphocytic leukaemia (CLL), one of hairy cell leukaemia, and 21 of other haematological diseases as shown in Table 1. The remaining 24 were without evidence of haematological disorders. The ages of the 80 patients ranged from 2 to 85 yr, and they included 43 males and 37 females.

IMMUNOCYTOCHEMISTRY FOR LACTOFERRIN

Intracellular lactoferrin was observed light microscopically with peroxidase-antiperoxidase (PAP) method as described by Mason.⁷ Rabbit specific antisera to human lactoferrin, swine antisera to rabbit immunoglobulins, and PAP complex were obtained from Dako-immunoglobulins Ltd, Copenhagen, Denmark. Control studies were carried out in the following two ways: one was negative control, substituting the first specific antisera with non-immunised normal rabbit serum, and the other was positive control, using peripheral blood smears from healthy volunteers. Endogenous peroxidase activity was blocked by immersing samples in methanol containing 0.3% hydrogen peroxide for 30 min. The specimens were observed after coun-

terstaining by use of Wright-Giemsa stain to identify each cell type precisely.

SCORING FOR LACTOFERRIN

The staining patterns and ratings of polymorphonuclear leucocytes for lactoferrin were classified as follows (see also Fig. 1):

Staining pattern	Rating
Type 0: negative	0
Type I: diffuse faint cytoplasmic reaction	1
Type II: focal intense cytoplasmic reaction	2
Type III: diffuse intense cytoplasmic reaction	3

The sum of the ratings of 100 polymorphonuclear leucocytes were defined as "lactoferrin-score" with a possible range from 0 to 300.

ULTRASTRUCTURAL CYTOCHEMISTRY FOR MYELOPEROXIDASE

Buffy coat specimens from two patients with acute myelogenous leukaemia were fixed in 2.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 30 min. After washing in the buffer, cytochemistry for myeloperoxidase was carried out according to modified Graham-Karnovsky's method,¹⁵ using 3, 3'-diaminobenzidine (DAB) as substrate. The samples were then postfixed in 1% osmium tetroxide for one hour, dehydrated in graded alcohols, and embedded in Epon by routine procedure. The ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate or lead citrate alone, and examined with a JEOL-100C electron microscope at an accelerating voltage of 80 kV.

Results

LIGHT MICROSCOPIC IMMUNOCYTOCHEMISTRY FOR LACTOFERRIN

Lactoferrin scores in examined cases are summarised in Fig. 2, and the cases, in which lactoferrin-negative polymorphonuclear leucocytes (type 0) were found, are summarised in Table 1.

In healthy volunteers or patients with non-haematological diseases, polymorphonuclear leucocytes showed diffuse intense cytoplasmic reaction for lactoferrin (type III) as has been observed by Mason.⁷ Cells after the metamyelocyte stage of maturation were positive for lactoferrin, but myelocytes were either positive or negative, and cells at the earlier stage of maturation were negative for lactoferrin. Lactoferrin-deficient polymorphonuclear leucocytes were rarely found in some of these patients, but lactoferrin-scores were almost normal in this group. Reaction products for lactoferrin were never

Fig. 1 Staining patterns and ratings of polymorphonuclear leucocytes for lactoferrin.

Fig. 2 Lactoferrin (LF) scores of examined cases. A dot represents one patient, and an open circle represents a case with LF-negative polymorphonuclear leucocytes. A solid line connects LF-scores on the different time points of one patient. (* = blast crisis; † = case 1; ‡ = case 2; 1 = APL; 2, 3 = AMoL; 4 = AMMoL; 5, 6 = CMMoL; 7, 8 = ALL; 9, 10 = CLL; 11 = hairy cell leukaemia.)

found in other blood cell lines, including eosinophils, basophils and monocytes. No endogenous peroxidase activity was observed in the negative control studies.

In each patient with haematological disease, most or all polymorphonuclear leucocytes stained positively for lactoferrin with the same pattern as those from healthy volunteers (type III). However, weak or negative reaction was found in some of the

polymorphonuclear leucocytes from certain leukaemic patients (type 0–II). Significantly low lactoferrin-scores were frequently found in AML, and lactoferrin-negative polymorphonuclear leucocytes were encountered in eight patients with AML (8/14). Except AML, lactoferrin-negative polymorphonuclear leucocytes were found in the following cases: one patient with CML in the stage of blast crisis (1/10 with CML), one patient with APL (1/1),

Table 2 Clinical features and haematological data from two acute myelogenous leukaemia patients with lactoferrin deficiency

Case	1	2
Age (yr)	28	66
Sex	Female	Male
Clinical course	Purpura and anaemia No infection	Preleukaemic state about three months duration before leukaemic transformation, pneumonia
Peripheral blood		(preleukaemic phase) (overt leukaemic phase)
RBC ($10^6/\mu\text{l}$)	184	120
PLT ($10^3/\mu\text{l}$)	2.5	7.0
WBC ($/\mu\text{l}$)	9,100	8,900
differential cell count	MB 12%, PM 1% M+MM+ST+PMN 51 Ba 1, LY 35 EB 11/100WBC	MB 37%, MM+ST+PMN 38 BA 3.5, LY 19.5 MO 2, EB 5/100WBC
Bone marrow	Hypercellular MB 8.2%, PM 7.0% M+ST+PMN 30.8 EO 1, LY 8.4 EB 42.2, OT 2.4	Normocellular MB 4.8%, PM 4.6% M+MM+ST+PMN 52.6 EO 2.6, BA 3.0 LY 5.0, MO 2.2 EB 24.8, OT 0.4
Cytochemistry for PMN	PO (-) 27% (+) 73% βG (-) 90% (+) 10% Ch-ES (-) 1% (+) 99% Ac-P (+) 100% LF (-) 95% (+) 5% LZ (+) 100% LAP score 131	ND PO (-) 40% (+) 60% βG (+) 100% Ch-ES (-) 80% (+) 20% Ac-P (+) 100% LF (-) 99% (+) 1% LZ (+) 100% LAP score 289

MB = myeloblasts; PM = promyelocytes; M = myelocytes; MM = metamyelocytes; ST = stabs; PMN = polymorphs; EO = eosinophils; BA = basophils; LY = lymphocytes; MO = monocytes; EB = erythroblasts; OT = others; PO = peroxidase; βG = beta glucuronidase; Ch-ES = naphthol-ASD-chloroacetate esterase; Ac-P = acid phosphatase; LF = lactoferrin; LZ = lysozyme; LAP = alkaline phosphatase (normal range 180-280); ND = not done.

Fig. 3 Bone marrow finding from a patient with AML (case 1), stained for lactoferrin. Polymorphonuclear leucocytes are all negative for lactoferrin except one (arrow). $\times 1600$.

one patient with AMoL (1/2), and one patient with CMMoL in a transitional phase to an AMMoL (1/2 with CMMoL). These cases are acute leukaemias and the acute phase of chronic leukaemias.

ULTRASTRUCTURE OF LACTOFERRIN-NEGATIVE POLYMORPHONUCLEAR LEUCOCYTES IN CASES OF AML

In two cases of AML (case 1 and 2 in Fig. 2), in which the majority of polymorphonuclear leucocytes were negative for lactoferrin, the ultrastructural morphology and cytochemistry for myeloperoxidase were studied. Clinical and haematological data on these cases are summarised in Table 2. In case 1, 95% of the patient's polymorphonuclear leucocytes were negative for lactoferrin (Fig. 3). These abnormal polymorphonuclear leucocytes contained decreased numbers of cytoplasmic granules, and specific granules were totally absent (Fig. 4). Primary granules were also decreased in number and showed varying degrees of myeloperoxidase deficiency. Case 2 developed an overt leukaemia after a pre-leukaemic state of three months duration. Ninety-nine percent of the patient's polymorphonuclear leucocytes lacked lactoferrin in the terminal phase. Ultrastructural analysis showed that specific granules were absent in these polymorphonuclear leucocytes (Fig. 5). Many of the granules present in this patient's polymorphonuclear leucocytes were elongated or spindle-shaped ranging from 0.45 μm to 1.50 μm in larger diameter and contained crystalline structure. These granules were considered as myeloperoxidase-deficient primary granules frequently seen in leukaemic cells^{16–20} and occasionally in hereditary anomalous neutrophils²¹ or normal immature neutrophils.²² The leucocyte alkaline phosphatase (LAP) scores were slightly decreased in case 1 and almost within normal range in case 2 (Table 2).

Discussion

The purpose of this study was to estimate the deficiency of lactoferrin in polymorphonuclear leucocytes semiquantitatively by means of a scoring technique and to clarify the ultrastructural abnormalities of lactoferrin-deficient polymorphonuclear leucocytes. In the present study, 35 cases of leukaemias, 21 cases of other haematological diseases and 24 cases of non-haematological diseases were examined with light microscopic immunoperoxidase method, and two cases of acute myelogenous leukaemia were studied with an electron microscope.

Lactoferrin-deficiency in polymorphonuclear leucocytes was frequently observed in acute

leukaemias in our study, whereas it was not found in cases of CML except in a case of blast crisis. This result confirmed the earlier report of Mason,¹⁰ who found lactoferrin-deficient polymorphonuclear leucocytes in four cases of acute or subacute myelomonocytic leukaemia, but not in CML. Previous ultrastructural studies of circulating polymorphonuclear leucocytes in leukaemic patients^{16,17} revealed abnormalities in granule populations, including absence of specific granules, in cases of AML and CML in blast crisis, but polymorphonuclear leucocytes in CML patients during chronic state were morphologically normal except some non-specific minor abnormalities. These data support our findings. On the other hand, Rausch *et al*¹¹ found lactoferrin-deficient polymorphonuclear leucocytes in two cases of acute leukaemias (2/7) and in three cases of CML (3/4) by the immunofluorescence method. Although our data cannot exclude the presence of lactoferrin-deficient polymorphonuclear leucocytes in CML, the discrepancy in cases of CML between two studies may partly derive from the different methods used: the immunoperoxidase and the immunofluorescence method. It is possible that eosinophils, which are negative for lactoferrin, were misinterpreted as lactoferrin-negative neutrophils by the immunofluorescence method. The immunoperoxidase method is more reliable than the immunofluorescence method for the identification of each cell type. However, as stated by Mason,¹⁰ the immunocytochemical method is basically semiquantitative, and a minor reduction of lactoferrin content in neutrophils may not be detectable by this method.

Morphological and cytochemical abnormalities in polymorphonuclear leucocytes have been reported in various disease states, such as congenital neutrophil anomalies^{12–14,21,23–28} as well as acquired disorders including leukaemias.^{10,11,16–20,29–32} Since lactoferrin-deficient polymorphonuclear leucocytes have been found in non-leukaemic conditions,^{12–14} deficiency of lactoferrin in neutrophils is not a specific finding for leukaemia. However, this study showed that distinctively low values of lactoferrin-score were found exclusively in cases of leukaemia. Therefore, it seems that low lactoferrin-scores may strongly suggest the presence of a leukaemic process, if not diagnostic. On the other hand, no significant differences of scores were found among acute leukaemias except two cases of AML (case 1 and 2) which showed the extremely low scores. These two cases had, however, no peculiar clinical findings (Table 2) as compared to other cases of AML, and scoring for lactoferrin had little diagnostic value for the subclassification of acute leukaemias.

Fig. 4 (a) Ultrastructure of a polymorphonuclear leucocyte from case 1, cytochemically stained for peroxidase. Specific granules are absent and some primary granules lack peroxidase activity (arrow) $\times 15\,000$; (b) Higher magnification of the cytoplasm of a polymorphonuclear leucocyte reacted for peroxidase. $\times 30\,000$.

Fig. 5 (a) Ultrastructure of a polymorphonuclear leucocyte from case 2, cytochemically stained for peroxidase. Specific granules are absent and peroxidase-positive primary granules show abnormal morphology. $\times 13\,000$. (b) Higher magnification of abnormal shaped primary granules, reacted for peroxidase. These granules are lacking peroxidase activity and contain crystalline internal structure. $\times 50\,000$.

The biological significance of lactoferrin deficiency in polymorphonuclear leucocytes in leukaemic patients is uncertain. Broxmeyer *et al*³³ have recently shown that lactoferrin is a physiological negative feedback regulator of granulopoiesis: a granulocytic "chalone". As lactoferrin deficiency was frequently observed in acute leukaemias, it may partly be involved in leukaemic progression of myeloid cells. However, contradictory data against this hypothesis has also been reported more recently;³⁴ and even if this hypothesis is correct, it is unlikely that leukaemic progression results from the deficiency of a single haemopoietic regulatory factor.

Breton-Gorius *et al*¹² and Boxer *et al*¹⁴ observed lack of specific granules in congenitally-lactoferrin-deficient polymorphonuclear leucocytes in patients with recurrent infections. In the present study, absence of specific granules was again demonstrated in lactoferrin-deficient polymorphonuclear leucocytes in leukaemic patients. These data suggest that lactoferrin is localised in specific granules of neutrophils. On the contrary, Parmley *et al*³⁵ have recently demonstrated lactoferrin in mature primary granules but not in specific granules of human neutrophils and rabbit heterophils with an immunoferritin method. In our cases, the following possibilities may also be considered: 1) lactoferrin deficiency has resulted from immaturity of primary granules in abnormal polymorphonuclear leucocytes, or 2) lactoferrin is deficient in spite of the presence of mature primary granules in these polymorphonuclear leucocytes. Further studies will be needed to confirm the precise localisation of lactoferrin and the mechanism of lactoferrin deficiency.

In electron microscopic observation, Bainton¹⁶ classified abnormal polymorphonuclear leucocytes in acute leukaemias into three categories: 1) polymorphonuclear leucocytes containing only azurophil granules and lacking specific granules, 2) polymorphonuclear leucocytes containing only specific granules and 3) polymorphonuclear leucocytes containing both types of granules but with the azurophil granules lacking peroxidase activity. Lactoferrin-deficient polymorphonuclear leucocytes in the present study correspond to the first category of Bainton's classification, but they also showed varying degree of peroxidase deficiency. This population of polymorphonuclear leucocytes may be a subtype of the first category: polymorphonuclear leucocytes containing only azurophil (primary) granules deficient in peroxidase.

Concerning the intracellular localisation of leucocyte alkaline phosphatase (LAP), there is no agreement among investigators. Bainton *et al*³⁶ observed

leucocyte alkaline phosphatase in specific granules of human myelocyte, whereas other workers found leucocyte alkaline phosphatase in plasma membrane fractions, in atypical granules or vesicles but not in specific granules.^{6,32,37-40} In our two patients with AML, in which almost all polymorphonuclear leucocytes were negative for lactoferrin and devoid of specific granules, scores for leucocyte alkaline phosphatase were only slightly decreased or almost within normal range. Furthermore, except in a case of blast crisis of CML, lactoferrin deficiency was not found in cases of CML with low scores for leucocyte alkaline phosphatase. These observations suggest that the localisation of these two proteins is different and that leucocyte alkaline phosphatase is at least localised in organelles other than specific granules.

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