Lactoferrin—Specific Localization in the Nuclei of Human Polymorphonuclear Neutrophilic Leukocytes (35779)

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Lactoferrin (LF) is a pink iron binding protein with a mol wt of 90,000, that was first isolated from bovine milk (1). It has been identified in the epithelial cells of a variety of glandular tissues, *e.g.*, breast, salivary glands and bronchial glands, and occurs in exocrine products such as milk, saliva, tears, cervical mucous, seminal fluid, bile, and bronchial secretions (2–4). Because of its high binding affinity for iron (5–6), it is bacteriostatic for microorganisms requiring iron for growth (3, 7). The presence of lactoferrin in these tissues and secretions suggests that the protein may have a role in protecting mucous surfaces from infection by iron-requiring microorganisms (8).

LF biosynthesis in human hematopoietic tissues has recently been described (8) and its presence in polymorphonuclear leukocytes (PMN) was demonstrated by direct extraction and by immunofluorescent staining of these cells. The latter study indicated that in methanol fixed bone marrow, lactoferrin was localized in the cytoplasm of PMN. In addition Baggiolini *et al.* (9) have reported that LF can be isolated from the secondary granular fraction of rabbit polymorphonuclear leukocytes.

During the course of an investigation of the presence and distribution of LF in patients with chronic mucocutaneous candidiasis, we found by immunofluorescent staining, as well as by direct extraction procedures, that LF was located in the nuclei of human PMN rather than in the cytoplasm of these cells.

Materials and Methods. Lactoferrin was obtained from human colostrum by chromatography on a DEAE-cellulose column equilibrated with 0.01 *M* phosphate buffer, pH 7.0. This fraction was treated with 55% saturated $(NH_4)_2SO_4$. The resulting supernatant containing lactoferrin was dialyzed and then lyophilized. This material, as well as a rabbit antibody to human colostrum, was a gift of Dr. M. Lamm. The rabbit anticolostrum antibody demonstrated a single line in immunoelectrophoresis to this lactoferrin and several lines to the whole colostrum, Fig. 1.

Rabbits were immunized in the footpads with 2 mg of LF in complete Freund's adjuvant, boosted subcutaneously with an additional 2 mg of LF in incomplete adjuvant, and bled 3 weeks following the last injection. Guinea pigs were immunized in a similar manner with 100 μg of LF. A goat antihuman LF antibody was a gift of Dr. John Robbins. Immunoelectrophoresis with these antisera yielded a single line with lactoferrin as well as with colostrum. In other experiments, incubation of the LF with ^{59}Fe ferrous citrate was carried out prior to gel diffusion with the goat and rabbit anti-LF antibodies. Subsequent radioautography of these (Fig. 2) plates demonstrated intense radioactivity at the site of the immune precipitate. Gamma globulin fractions of these antisera were conjugated with fluorescein isothiocyanate and passed through Sephadex G-25 and DEAE-cellulose as previously described (10). These fluoresceinated antibodies were used for direct immunofluorescent studies.

Peripheral blood finger stick smears were obtained from 20 normal adults and patients with the following illnesses: Chediak-Higashi syndrome, chronic granulamatous disease, cyclic neutropenia, chronic neutropenia, chronic myelogenous leukemia, acute lymphocytic leukemia, acute myelogenous leukemia, and mucocutaneous candidiasis. Bone marrows were obtained by aspiration and the spicules were smeared directly without anti-

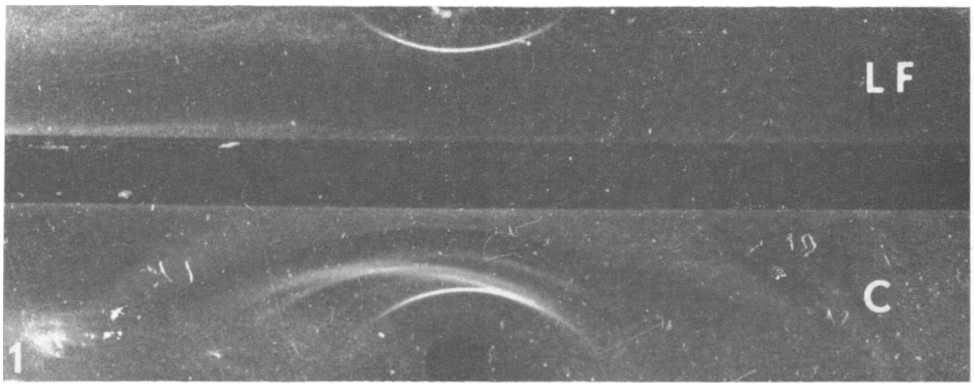

FIG. 1. (upper well) Purified lactoferrin (LF); (lower well) colostrum (C); (center trough) a rabbit antilactoferrin antibody. The antibody detects many antigens in colostrum and only a single antigen in the purified lactoferrin.

coagulation. Smears were air dried and then routinely fixed for 10 min with 95% ethanol. In selected cases acetone, *n*-propanol, or absolute methanol were employed as the fixatives. The slides were washed in phosphate buffered saline, pH 7.4 (PBS), then covered with fluorescein-labeled rabbit antilactoferrin antibody. After 30 min they were rinsed in PBS and mounted in buffered glycerol, pH 7.2.

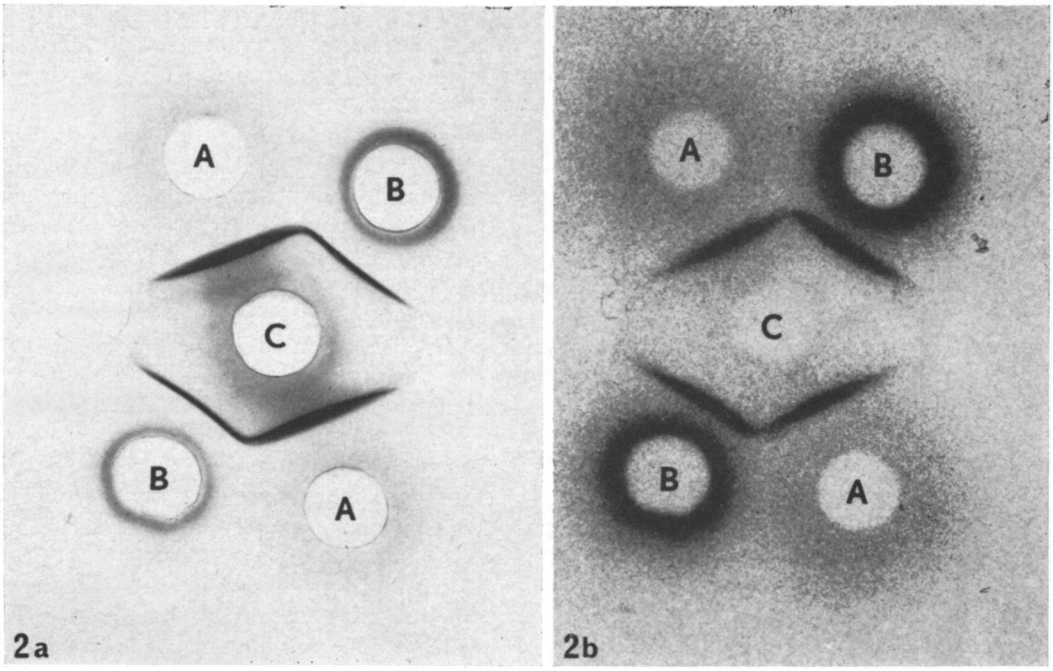

FIG. 2a. Gel diffusion analysis: (C) goat anti-LF antibody; (A) LF isolated from colostrum; and (B) human milk. A line of identity is formed between the precipitin lines. (b) Radioautograph of same gel diffusion plate demonstrating intense radioactivity of the immune precipitates. This analysis demonstrates the identity between LF isolated from colostrum and LF present in whole milk and the Fe binding property of this protein.

Indirect (sandwich) immunofluorescent studies were also carried out by exposing blood smears to 1:10 dilutions of either normal guinea pig sera or guinea pig anti-LF antisera. The slides were then washed in PBS and exposed to a fluoresceinated rabbit antibody to the Fab portion of guinea pig immunoglobulin. They were again washed in PBS and mounted in glycerol. The slides were then examined by ultraviolet light using a Leitz Ortholux microscope. Photographs were taken with a Leitz Orthomat camera using Kodak Tri-X film.

Results. Direct immunofluorescent staining employing either fluoresceinated rabbit or goat anti-LF antibodies of blood or bone marrow smears fixed in ethanol, *n*-propanol, or acetone demonstrated that specific fluorescence was localized to the nuclei of all band forms and mature polymorphonuclear leukocytes (Fig. 3a). Often the staining appeared to be in the nuclear membrane (Fig. 3b). Cells from all of the normal volunteers as well as all patients studied, demonstrated that LF was localized in the nuclei of PMN.

Neither nuclear nor cytoplasmic specific staining was observed in lymphocytes, monocytes, or eosinophiles (Fig. 3c). Bone marrow preparations showed no staining of promyelocytes, myelocytes (Fig. 3d), lymphoblasts, normoblasts, or leukemic lymphoblasts, or myeloblasts. In one patient with chronic myelogenous leukemia in relapse, LF was found to be present only in the nuclei of band forms and mature PMN. Occasional nonspecific staining was observed in the granules of eosinophilic polymorphonuclear leukocytes. Peripheral blood and bone marrow smears stained with fluoresceinated rabbit antibodies of other specificities, *e.g.*, antibovine serum albumin, never demonstrated any staining of cells except the nonspecific staining of eosinophile granules.

Indirect immunofluorescent stains with blood smears fixed in ethanol or acetone and then treated with guinea pig anti-LF antibodies or normal guinea pig serum followed by the fluorescein-labeled rabbit anti-guinea pig Fab fragment antibody demonstrated specific staining of PMN nuclei only when the guinea pig anti-LF antibody was used in the first step of the procedure.

Blood and bone marrow smears fixed in methanol and subjected to direct immunofluorescent staining for lactoferrin distribution showed staining in both the nucleus and cytoplasm; this staining was also confined to band forms and mature polymorphonuclear leukocytes. It was also noted that slides fixed in ethanol and stored at 4° for even a few days lead to an increase in cytoplasmic staining of the PMN.

Because the variation in the localization of LF appeared to depend on the fixatives used prior to immunofluorescent staining an attempt was made to isolate LF directly isolated PMN nuclei. Eighty ml of blood was obtained from normal adults, was anticoagulated with 10 mg of heparin and sedimented with 20 ml of dextran (mol wt 200,000). The plasma was spun at 1000 rpm for 10 min to obtain the cells, these were washed once in Eagles medium and then incubated for 10 min in hypotonic buffer, 0.01 *M* NaCl, 0.01 *M* Tris, and 0.0015 *M* $MgCl_2$ (11) and dounced 20 times with a 7-ml glass homogenizer. The broken cells were then washed twice by layering on, and spinning through, fetal calf serum. A small aliquot of these pellets were smeared from calf serum, fixed in ethanol or methanol, and stained with the fluoresceinated goat anti-LF antibody. The remainder of the pellet was frozen thawed 6 times and extracted overnight at 4° with 2 ml of 1 *M* NaCl (9). Light microscopic examination of Giemsa stained smears of the dounced and washed cells prior to freeze thawing demonstrated free PMN nuclei, often in clumps as well as lymphocyte nuclei; no PMN cytoplasm was seen. The extract was concentrated to 1 ml by pressure dialysis. The presence of LF in the extract was detected by gel diffusion against a rabbit anti LF antibody (Fig. 4) and also demonstrated by the ability of these extracts to inhibit the binding of ^{125}I lactoferrin by a goat antilactoferrin antibody, Table I.

Immunofluorescent staining of these isolated nuclei again demonstrated bright specific staining in the nuclei of PMN, but no staining was observed in lymphocyte nuclei. In the smears of isolated nuclei fixed in

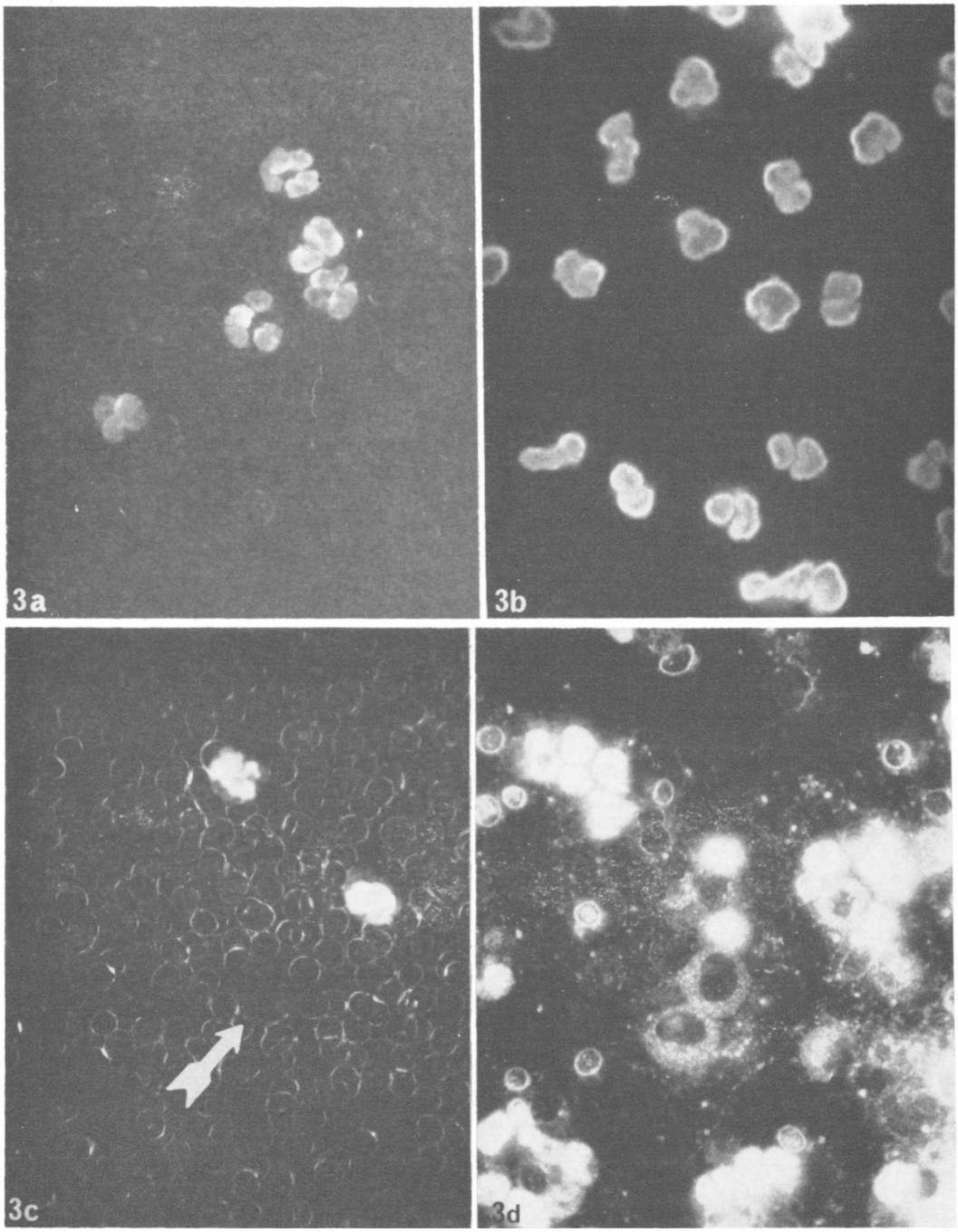

FIG. 3a. Peripheral blood smear: nuclei of 4 PMN show bright immunofluorescent staining; no staining is observed in cytoplasm; 400×. (b) Buffy coat preparation from peripheral blood: Here staining is more intense at nuclear membrane; again cytoplasmic staining is absent; 400×. (c) Peripheral blood smear: Nuclei of 2 PMN stain intensely; (arrow) an unstained eosinophile. Partial illumination with tungsten light makes the red blood cells visible; 400×. (d) Bone marrow smear: Nuclei of mature PMN stain intensely; (just below center) two myelocytes. Cytoplasmic granules can be seen because of partial illumination with tungsten light. Nuclei of these cells demonstrates absence of staining for LF; 400×.

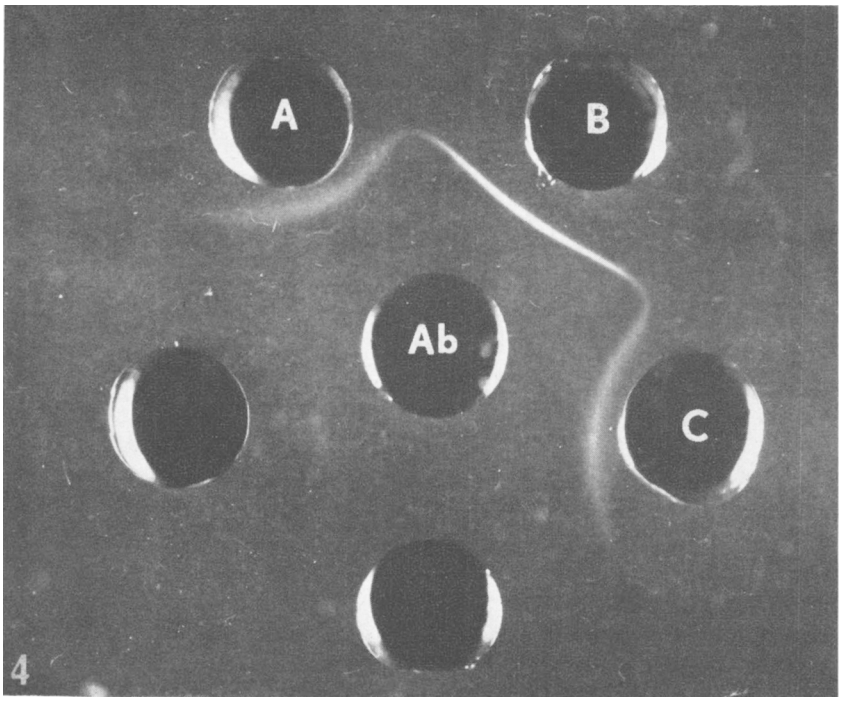

FIG. 4. Gel diffusion analysis: (Ab) contains goat anti-LF antibody; (B) LF, 250 μg/ml. (A, C) nuclear extract (see text) from two normal adults. A line of identity forms between the immune precipitates.

methanol there was also staining of the PMN nuclei; however there was a consistently higher diffuse background staining between these nuclei, as well as of other non-PMN nuclei.

Discussion. This study demonstrates that an iron binding protein, lactoferrin, is present in the nuclei and/or on the nuclear membranes of mature polymorphonuclear neutrophilic leukocytes; it is absent from other types of cells found in peripheral blood or bone marrow. Our findings are therefore in general accord with those of Masson *et al.* (8) and Baggiolini *et al.* (9); however, the subcellular location of LF reported by these authors is cytoplasmic rather than nuclear. Since, in the latter study, LF was extracted from a cytoplasmic granule of rabbit polymorphonuclear leukocytes, a species difference may in part explain the difference in location. However, preliminary immunofluorescent studies demonstrate LF to be present in the nuclei of bovine polymorphonuclear leukocytes. When methanol is used as a fixative, as in the studies of Masson *et al.* (8) cytoplasmic localization of LF is predominant. Although the precise mechanisms by which different fixatives give different cytological localization have not been explained, it is hard to conceive of an artifact that would lead to strictly nuclear localization of LF. Furthermore, LF can be located by immunofluorescence in isolated PMN nuclei and can be directly extracted from these nuclei.

Since LF is present only in band forms and mature PMN but not in myeloid precursors, its accumulation is probably a late feature of PMN maturation similar to the development of secondary granules and glycogen formation (12–13). LF is thus a unique chemical marker of PMN nuclear maturity. Of interest in this regard is that the leukemic neutrophiles of a patient with chronic myelogenous leukemia, which lack the secondary granule enzymes such as alkaline phosphatase (14), stained normally for LF. Also in the Chediak-Higashi syndrome in which lysosome formation is abnormal (15), the

TABLE I. Inhibition of Binding of ^{125}I Lactoferrin by Extract of Nuclear Fraction of Peripheral Blood Leukocytes.

% Binding of ^{125}I lactoferrin[a] in presence of 0.1 ml of		Inhibition (%)
PBS	67.9	
Extract	8.5	95.6
Lactoferrin, 5γ in 0.1 ml	3.4	>100
Lactoferrin, .5γ in 0.1 ml	53.7	23.0

[a] 0.1 ml of ^{125}I LF (10 μg/ml) mixed with 0.1 ml of PBS, the extract, or LF standards; 0.1 ml of antibody (1:500 dilution of goat anti-LF antibody in 50% normal guinea pig serum) was added. Mixture was incubated for 30 min and then 0.6 ml of 66% saturated $(NH_4)_2SO_4$ was added. After 60 min at 4° the tubes were spun and a 0.2-ml aliquot of the supernatant was counted in a gamma-ray spectrometer. Control samples were prepared containing 0.1 ml of ^{125}I LF, 0.2 ml of PBS, and 0.6 ml of 66% saturated $(NH_4)_2SO_4$; 0.2 ml of the control was also counted. The percentage of binding was calculated from the ratio: [1—(counts in experimental samples/counts in control samples)] × 100. The percentage inhibition of binding of ^{125}I lactoferrin is calculated from the formula:

[binding in presence of PBS—binding with inhibitor (extract or lactoferrin standards)]/[binding in presence of PBS—binding of ^{125}I lactoferrin by 50% normal guinea pig serum (this value was 5.8%)].

PMN showed normal nuclear staining for LF and the cytoplasmic giant lysosomes of these cells did not stain for LF. Thus in two diseases where lysosome formation is abnormal, LF was present in the nucleus in grossly normal amounts. This finding supports the view that LF is not lysosome-associated in humans.

The presence of LF in many types of epithelial cells and their exocrine products have suggested that it may have a role in defense against those microorganisms requiring iron for their growth. In a preliminary effort to identify a role for LF, we examined blood smears from patients with apparent increased susceptibility to infections, with unexplained fevers, as well as a variety of fungal infections. In all of these patients, LF was nuclear and not reduced in quantity as determined by immunofluorescent staining.

The presence of LF in PMN nuclei for the purpose of bacteriostasis by iron chelation would appear rather inefficient unless the LF could be released from its nuclear location and/or if the PMN degenerated and thus released its nuclear LF at the site of bacterial invasion. It has been recently demonstrated that iron is present in the nuclei of nonhematological cells (Hela cells) (16), here iron appears to be necessary for cell replication. These considerations suggest that LF present in the nuclei of mature human PMN may be involved in some as yet obscure, but physiologically important role.

Summary. Lactoferrin, a pink iron binding protein first isolated from bovine milk has been found in the nuclei and/or nuclear membrane of mature polymorphonuclear neutrophilic leukocytes. It is absent from all other cells of the hematopoietic system. Grossly normal amounts of lactoferrin were found to be present in the neutrophiles from patients with a variety of hematological abnormalities.

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