# Association of Lactoferrin with Lysozyme in Granules of Human Polymorphonuclear Leukocytes

MARY S. LEFFELL AND JOHN K. SPITZNAGEL

*Department of Bacteriology and Immunology, School of Medicine, University of North Carolina,
Chapel Hill, North Carolina 27514*

Lactoferrin is contained in cytoplasmic granules of human polymorphonuclear leukocytes. Upon centrifugation, it sediments in a band of granules that also contain 50% of the lysozyme activity. This granule class is distinct from others associated with alkaline phosphatase and peroxidase. The granules are latent for lactoferrin as only lysed granules have the capacity specifically to inhibit antigen binding by anti-lactoferrin serum.

Recently lactoferrin, an iron-binding protein, has been identified as a component of human polymorphonuclear leukocytes (PMN) (6, 13). Since lactoferrin possesses bacteriostatic action (11, 14), it could function, if it is a lysosomal protein, as an antibacterial agent in the phago-lysosomes of PMN. Baggiolini et al. recently demonstrated with rabbit PMN that lactoferrin is associated with alkaline phosphatase and lysozyme in specific lysosomal granules (1). But for human PMN the localization of this protein has not been resolved. Masson et al. have reported a cytoplasmic localization, whereas Green et al. have shown specific localization of lactoferrin only in or on the nuclei of PMN (6, 13). We report here for human PMN that lactoferrin is associated with a class of lysosomal granules also rich in lysozyme. Its possible function as an antibacterial agent is discussed.

## MATERIALS AND METHODS

**Preparation of granules.** Leukocytes obtained from 400 ml of freshly drawn, heparinized blood by dextran sedimentation yielded suspensions that comprised 90 to 95% PMN. In some experiments, cells were further purified to 95 to 98% PMN on a Ficoll-Hypaque gradient, as described by Böyum (3). This step removed monocytes, lymphocytes, basophils, and platelets from the granulocytes. Homogenized in sucrose and centrifuged at 126 $\times$ $g$ for 15 min, these cells yielded a granule-rich supernatant fluid and a pellet of nuclei and unbroken cells. Details of the homogenization procedure have been previously described (16). The granule suspension was then centrifuged through a sucrose density gradient under these conditions: $_0\int^t \omega^2 dt = 2.3 \times 10^{10} \sec^{-1}$, $R_{min} = 6.4$ cm, $R_{max} = 15.3$ cm. The gradient volume was 50

ml, and the gradient was linear from 30 to 53% sucrose, except for a loading volume of 2.7 ml of 11% sucrose. Twenty 2.7-ml fractions were collected from each gradient. Protein, lysozyme, and peroxidase activity were determined as previously described (16). Alkaline phosphatase activity was determined using $p$-nitrophenyl phosphate as the substrate in a 0.1 M diethanolamine buffer as described by Baggiolini (2). Polyacrylamide gel electrophoresis was done with a $p$H 4.5 buffer and 7.5% polyacrylamide gels; a stacking gel was not used.

**Measurement of lactoferrin.** Lactoferrin was purified from human milk by the method of Masson and Heremans (12). Rabbits were immunized in the hind foot-pads with 2 mg of this purified protein in complete Freund adjuvant, followed in 2 weeks by a booster injection of 2 mg in incomplete Freund adjuvant subcutaneously. Bleeding was by cardiac puncture 3 weeks after the final injection. This antiserum gave a single precipitin line when reacted with the crude milk proteins in immunoelectrophoresis. Both the purified milk lactoferrin and rabbit antiserum were further checked for purity against similar preparations kindly donated by J. Bienenstock and T. B. Tomasi, Jr. With the milk lactoferrin as a standard, quantitation of the various PMN fractions was done by single, radial immunodiffusion, as described by Mancini (10). To obtain maximum recovery, it was necessary to prepare the agarose-antibody mixture in a 0.1 M phosphate buffer with 1 M NaCl at $p$H 8.0 and to extract the granules in 0.3% cetyltrimethylammonium bromide (CTAB, Eastman Kodak). In some cases, extractions were made with 0.1% Triton X-100 (Sigma Chemical Co.).

**Latency assay.** The inclusion of an enzyme in PMN granules is routinely determined by demonstration of enzyme latency. An enzyme is said to be latent, and thus included in PMN granules, if full recovery of enzymatic activity depends upon detergent lysis of the

granules. Lacking an enzymatic assay, the latency of the lactoferrin granules was measured by the ability of lysed or whole granules to inhibit antigen binding as measured by the Farr ammonium sulfate precipitation method (5). Since lactoferrin is soluble in 50% ammonium sulfate, it is a suitable antigen for this assay. Milk lactoferrin was labeled with [125]I (ICN, Tracerlab) by the MacFarlane monochloride method (9). A standard dilution of the anti-lactoferrin serum was mixed with solutions of either whole or lysed granules at 4 C. The granules and lysed debris were immediately removed by centrifugation at 3,000 × g for 10 min. The reduction in the ability of the serum to bind ^{125}I-lactoferrin was then measured.

## RESULTS

Sixty-six percent of the lactoferrin was confined to a single class of granules. Resolution of the granules on the gradient produced at least three bands, as shown in Fig. 1. These bands are marked by the positions of alkaline phosphatase, lactoferrin, and peroxidase. Electron microscope examination has verified the presence of membrane-bounded particles, or granules, in these bands. Lactoferrin was found in the middle band of granules at a sucrose concentration of 37 to 39%. Lysozyme appears to have a bimodal distribution; however, the middle or lactoferrin band usually contained at least 50% of the lysozyme activity. It is apparent that the band of granules containing the lactoferrin and half of the lysozyme activity is distinct from those associated with alkaline phosphatase and peroxidase.

The presence of lactoferrin in the granule extracts was confirmed by the following criteria. These extracts contained a protein with electrophoretic mobility and immunological reactivity identical to purified milk lactoferrin; this protein, unlike serum transferrin (8), bound ^{59}Fe even after dialysis at pH 4.0. Disc gel electrophoresis at pH 4.5 of extracts from the gradient lactoferrin peak showed two major components—a more anodal band migrating to the same position as milk lactoferrin, and a cathodal band having lysozyme activity (Fig. 2). The larger anodal protein band reacted specifically with anti-lactoferrin serum and bound [59]Fe.

Lactoferrin was present in high specific concentrations in the PMN granule fractions, as shown in Table 1, which suggests that it is indeed a granular component. Its specific concentration in the whole-cell homogenates and the granule suspension is approximately two times greater than that of the nuclear pellet. Moreover, resolution of the granules on the gradient produces a further twofold increase in the specific concentration. As reported by others (6, 13) lactoferrin was undetectable in monocyte-enriched fractions
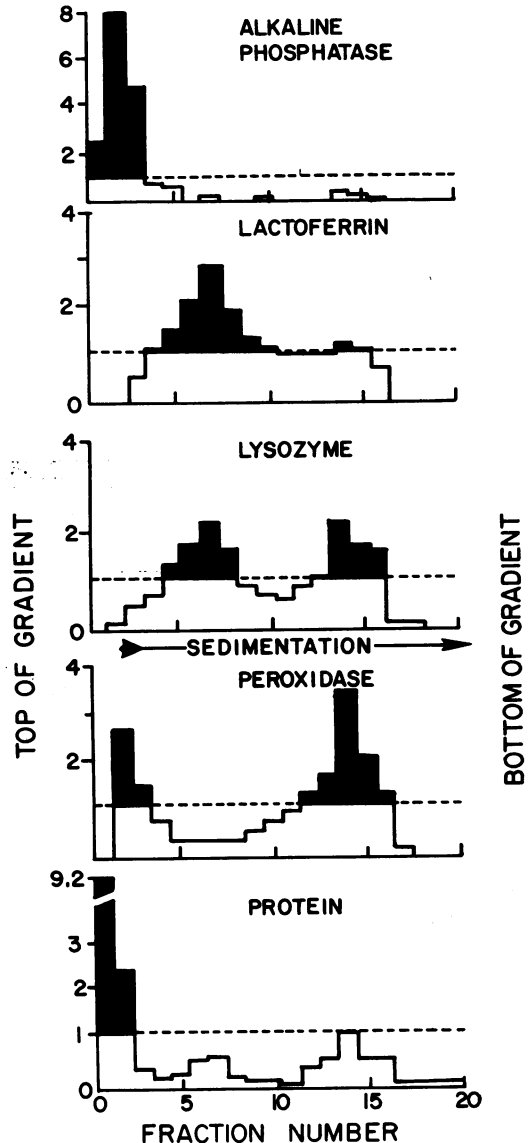


FIG. 1. *Fractionation of leukocyte granules by sucrose density centrifugation. Graphs are normalized distribution histograms as a function of the total protein or total enzymatic activity recovered. Ordinate is concentration in fraction relative to concentration corresponding to uniform distribution throughout the gradient. Percentage recoveries were 92 for alkaline phosphatase, 69 for lactoferrin, 74 for lysozyme, 83 for peroxidase, and 87 for protein.*

from the Ficoll purification. It was present, however, in PMN at a concentration of 5 to 6 μg/10$^6$ cells, a figure which compares well with the one in the literature (13).

As Masson et al. have demonstrated, lacto-

ferrin, owing to its relatively high isoelectric point, is easily absorbed on acidic macromolecules and, therefore, must be extracted at either low pH or high ionic strength (13). By the same criterion, it seemed possible that, rather than being included in granules, it might merely be absorbed to their surfaces. To test this possibility, we attempted to demonstrate latency for the lactoferrin in granules. Thus, if lactoferrin was included in the granules, absorption of an anti-lactoferrin serum with whole granules should not reduce the serum antigen-binding capacity. Lysis of the granules with detergents should free lactoferrin for inhibition of the antigen-binding capacity. Alternatively, if it is present on the surfaces of the granules, inhibition should be



FIG. 2. *Comparison of purified milk lactoferrin (M) with lactoferrin granule extracts (L) by polyacrylamide gel electrophoresis. Gels were stained with amido schwarz. Duplicate unstained granule extract (L') shows pin marking the area of gel reacting with lysozyme substrate plate. Migration was toward the cathode at the bottom of the gels.*

observed with or without granule lysis. Figure 3 shows the results from such an experiment. The serum dilution of the rabbit anti-lactoferrin used was capable of binding 99% of the $^{125}$I-lactoferrin. A background inhibition was seen with sucrose, CTAB, or Triton X-100 alone, and thus the results are expressed as percentages of the inhibition observed above background. When dilutions of a freshly prepared granule suspension in sucrose (whole granules) were used to absorb the anti-lactoferrin serum, no inhibition above background was observed at any concentration, compared to the titrable inhibition seen with the same granules diluted in CTAB or Triton. As might be expected, the more anionic detergent, CTAB was more effective than Triton X-100 in freeing lactoferrin for inhibition. Clearly these granules appear latent for lactoferrin.

## DISCUSSION

These experiments confirm the earlier work of Masson et al. that lactoferrin is a cytoplasmic component of human PMN (13). Moreover, they provide direct evidence that lactoferrin is included in a class of lysosomal granules associated with lysozyme. This finding correlates with the results of Baggiolini et al. who found lactoferrin in specific granules associated with 60% of the lysozyme activity (1). Unlike the rabbit, however, lactoferrin in human PMN granules is not associated with alkaline phosphatase.

Green et al. reported that the specific localization of lactoferrin in the nuclei of PMN was dependent upon the fixative used prior to immunofluorescent staining (6). In our hands, the lactoferrin granules were clearly latent; however, they were also somewhat labile. We found it necessary to use freshly prepared granules, because, even upon standing, some lactoferrin could be released into the supernatant fluid. In view of its affinity for acidic macromolecules, such as nucleic acids, it may be that the nuclear
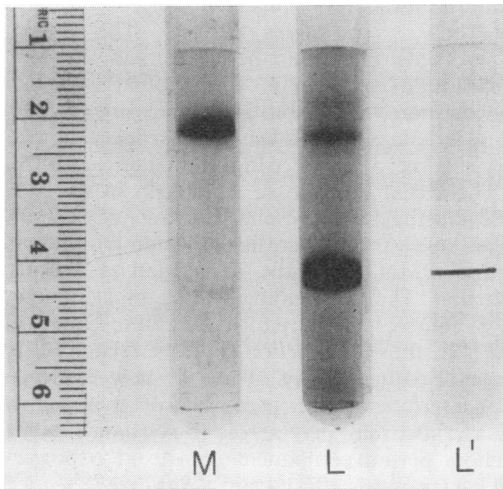
TABLE 1. *Specific concentration of lactoferrin (LF) in fractions of human heterophil leukocytes*

| No. of gradients | Cell suspension | Whole-cell homogenates | 126 × g Pellet | 126 × g Supernatant fluid | Gradient LF peak | Micrograms of LF /10⁶ cells |
|---|---|---|---|---|---|---|
| 4 | Whole blood leukocytes (90–95% PMN) | 121.4 ± 32.4[a] | 74.2 ± 6.05 | 115.3 ± 20.1 | 278.8 ± 98.1 | 6.4 ± 1.82 |
| 5 | Ficoll-purified PMN (95–98% PMN)[b] | 126.6 ± 55.3 | 59.5 ± 9.9 | 132.3 ± 49.7 | 280.6 ± 62.5 | |

[a] Results expressed as micrograms of LF per milligram of protein, mean ± standard deviation.
[b] No LF could be detected in monocyte-enriched fractions from Ficoll purifications.
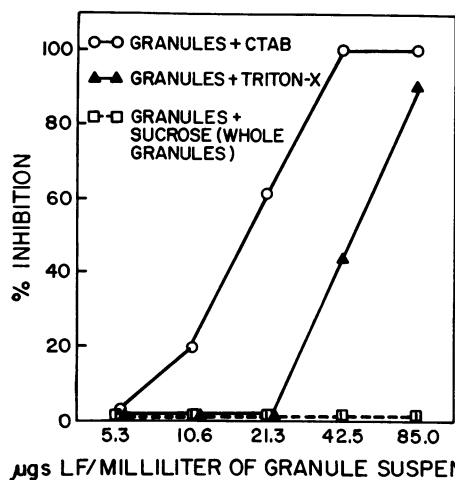
μgs LF/MILLILITER OF GRANULE SUSPENSION

FIG. 3. *Inhibition of antigen-binding capacity of rabbit anti-lactoferrin serum for $^{125}I$-lactoferrin by lysed granules. Results are expressed as percentages of the inhibition observed above background. Concentrations of lactoferrin in the inhibiting suspensions were independently determined by radial immunodiffusion.*

localization reported for lactoferrin is an artifact of fixation and staining. It was also reported that PMN from a patient with chronic myelogenous leukemia, which lack alkaline phosphatase, stained normally for lactoferrin. As alkaline phosphatase is considered an enzyme marker for secondary granules, this observation was argued as evidence for the noninclusion of lactoferrin in granules (6). Since our data show that lactoferrin and alkaline phosphatase are in different granule classes, it appears that such PMN could be deficient in the alkaline phosphatase granule class and yet normal with respect to the lactoferrin granules.

Our experiments do not completely exclude the possibility of the association of lactoferrin with a nuclear fraction. The 126 × *g* nuclear pellet separated from whole-cell homogenates contains a significant amount of lactoferrin; however, this fraction also contains many unbroken cells and much debris. Lactoferrin would be expected in this subcellular fraction. What is important is that the specific concentration of lactoferrin increases, first, with the separation of the granule fraction, and, secondly, with the resolution of a single granule class on the gradient, suggesting the purification of the subcellular source of lactoferrin.

The actual inclusion of lactoferrin in the granules was demonstrated by the latency experiments. Since only lysed granules inhibited antigen binding, it was argued that lactoferrin must be included in the granules and not merely absorbed

to their surfaces. It is possible that lactoferrin could be surface-bound in such a way that the binding sites between granules and the lactoferrin molecule could sterically block the antigenic binding. To explain the complete lack of inhibition shown by whole granules, two additional assumptions must be made to support this argument. First, the lactoferrin molecule must react as if it were antigenically univalent, and, secondly, the granule-binding sites and antigenic sites should be closely adjacent, if not identical. Under any other conditions, at least some intermediate binding would be expected. Lactoferrin is a large enough protein, with a molecular weight of approximately 80,000 to 90,000 (12), to have more than one antigenic site; moreover, its facility to precipitate in Ouchterlony gels argues against univalency. Thus, the concept of steric hindrance seems highly unlikely. The latency experiments, then, must be taken to show actual inclusion of lactoferrin in lysosomal granules. Furthermore, the quantitative data suggest that these granules are at least the primary, if not sole, source of lactoferrin within human PMN.

The actual function of lactoferrin in PMN is still undefined. Our results and those of Masson et al. suggest that there is a substantial amount within PMN, sufficient to suggest a specific function. The outstanding feature of lactoferrin is, of course, its affinity for binding Fe, as well as Cu (12); thus, its function could be related to its metal-chelating ability. There is now evidence that interference with bacterial iron metabolism by the host may play a role in resistance to infection. Serum components can exert a powerful bacteriostatic effect against *Pasteurella septica* and *Clostridium welchii* type A, an effect which can be abolished by the addition of Fe (4, 15). For *Clostridium*, the bacteriostatic serum agent has been identified as the iron-binding protein transferrin, acting with a $beta_2$ or gamma globulin (15). Lactoferrin is also supposed to be antibacterial against microorganisms requiring Fe and may, thus, function similarly to serum transferrin in cellular defense against infection. It is, in fact, only bactericidal at high concentrations (14); this concentration requirement, however, might be met within the phagocytic vacuole. Still it need not be directly bactericidal for its presence to be beneficial. During various infections and toxemic states, iron is deposited within reticuloendothelial cells, and it is further known that iron is inhibitory to certain bacterial endo- and exotoxins (7). Alternative functions for lactoferrin could then be in iron metabolism as a chelator of excess iron or in antibacterial activity as a source of Fe for toxin inactivation. Conceivably, lactoferrin could also synergistically

enhance the antibacterial effects of other lyso-somal components.

## LITERATURE CITED

1. Baggiolini, M., C. deDuve, P. L. Masson, and J. F. Heremans. 1970. Association of lactoferrin with specific granules in rabbit heterophil leukocytes. J. Exp. Med. 131:559–570.
2. Baggiolini, M., J. G. Hirsch, and C. deDuve. 1969. Resolution of granules from rabbit heterophile leukocytes into distinct populations by zonal sedimentation. J. Cell Biol. 40:529–541.
3. Böyum, A. 1968. Isolation of mononuclear cells and granulo-cytes from human blood. Scand. J. Clin. Lab. Invest. 21(Suppl. 97):77–89.
4. Bullen, J. J., H. J. Rogers, and J. E. Lewin. 1971. The bacterio-static effect of serum on Pasteurella septica and its abolition by iron compounds. Immunology 20:391–406.
5. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. J. Infect. Dis. 103:239–262.
6. Green, I., C. H. Kirkpatrick, and D. C. Dale. 1971. Lacto-ferrin-specific localization in the nuclei of human poly-morphonuclear neutrophilic leukocytes. Proc. Soc. Exp. Biol. Med. 137:1311–1317.
7. Janoff, A., and B. W. Zweifach. 1960. Inactivation of bacterial exotoxins and endotoxin by iron. J. Exp. Med. 112:23–34.
8. Laurell, C. B. 1947. Studies on the transportation and metabolism of iron in the body. Acta Physiol. Scand. 14(Suppl. 46):1–129.
9. MacFarlane, A. S. 1958. Iodine monochloride iodination of proteins. Nature (London) 182:53.
10. Mancini, G., O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235–254.
11. Masson, P. L., and J. F. Heremans. 1966. Studies on lacto-ferrin, the iron-binding protein of secretions. Protides Biol. Fluids Proc. Colloq. Bruges 14:115–124.
12. Masson, P. L., and J. F. Heremans. 1968. Metal-combining properties of human lactoferrin (red milk protein). 1. The involvement of bicarbonate in the reaction. Eur. J. Biochem. 6:579–584.
13. Masson, P. L., J. F. Heremans, and E. Schonne. 1969. Lactoferrin, an iron-binding protein in neutrophilic leuko-cytes. J. Exp. Med. 130:643–658.
14. Oram, J. D., and B. Reiter. 1968. Inhibition of bacteria by lactoferrin and other iron-chelating agents. Biochim. Biophys. Acta 170:351–365.
15. Rogers, H. J., J. J. Bullen, and G. H. Cushnie. 1970. Iron compounds and resistance to infection. Further experiments with Clostridium welchii type A in vivo and in vitro. Im-munology 19:521–538.
16. Welsh, I. R. H., and J. K. Spitznagel. 1971. Distribution of lysosomal enzymes, cationic proteins, and bactericidal substances in subcellular fractions of human polymorpho-nuclear leukocytes. Infect. Immunity 4:97–102.