

## ASSOCIATION OF LACTOFERRIN WITH SPECIFIC GRANULES IN RABBIT HETEROPHIL LEUKOCYTES\*

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Lactoferrin is an iron-binding protein which occurs in milk and in many other biological fluids, such as tears, saliva, nasal and bronchial secretions, gastrointestinal juice, seminal fluid, cervical mucus, and urine, but not in serum (1, 2). It has been found also in epithelial cells of glandular acini from human bronchi (3) and submaxillary glands (4), and is actively synthesized in tissue culture by human rectal mucosa, uterus, and kidney (2). It shares with transferrin the ability to bind reversibly two atoms of iron per molecule (5, 6), but differs from the serum protein by its antigenic properties (7, 8) and by its affinity for iron (7-10).

Lactoferrin has been recently detected in human and guinea pig heterophil leukocytes (11). These cells differ from the other known sources of lactoferrin in that they do not appear to carry out a true secretory function. However, they contain abundant cytoplasmic granules which are discharged into phagocytic vacuoles upon the onset of phagocytosis (12-14). These granules form two main groups that are synthesized at different stages in the maturation of the cells, and can be recognized by their cytochemical staining properties (15-20).

The biochemical characterization of rabbit heterophil leukocyte granules, after their separation by zonal differential sedimentation (21) and by isopycnic density gradient centrifugation (22, 23), has recently been described. Altogether, four distinct particle populations could be distinguished in these investigations: (a) large, dense granules, corresponding to the primary or azurophil granules, and characterized by myeloperoxidase, a variety of acid hydrolases, and one-third of the lysozyme activity of the cells; (b) smaller, less dense granules, identified as the secondary or specific granules, containing the bulk of the alkaline phosphatase activity and two-thirds of the total lysozyme activity, but apparently devoid of acid hydrolases; (c) particles of distinctly smaller size and of even lower density than the two previous groups, possibly corresponding to the tertiary granules of Wetzel et al. (15, 17, 18), and containing a number of acid hydrolases, but no myeloperoxidase; (d) a membrane fraction of low density, characterized by a high content in acid *p*-nitrophenyl phosphatase activity.

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These findings have opened the possibility of investigating the intracellular localization of lactoferrin in heterophil leukocytes. This study forms the object of the present paper. It required the prior demonstration that lactoferrin is present also in heterophil leukocytes from rabbits, the only species for which good separation techniques are available, and that it can be determined on this material by means of previously developed immunological and electrophoretic techniques (11).

#### *Materials and Methods*

*Collection of Material.*—Rabbit heterophil leukocytes were collected from peritoneal exudates, following the procedure of Hirsch (24). Homogenization of the cells and preparation of the cytoplasmic suspension that served as starting material for the fractionation experiments were done as described before (21).

*Fractionation Procedures.*—Zonal differential centrifugation<sup>1</sup> was performed, as described by Baggiolini et al. (21), under conditions optimal for the separation of azurophil and specific granules. Isopycnic centrifugation was carried out in the Beaufay rotor (22, 23), from the following initial conditions: 14 ml of starting material brought to a density of 1.10 with sucrose, layered on top of a sucrose density gradient (19 ml), extending linearly with respect to volume between densities 1.18 and 1.32, and itself resting on a sucrose cushion (6 ml) of density 1.32. Centrifugation was performed for 60 min at 35,000 rpm, which sufficed to bring all the particles practically to their equilibrium position in the gradient. Details concerning the operation of the rotor have been given by Beaufay (25) and by Leighton et al. (26).

*Biochemical Assays.*—Protein, peroxidase, and acid and alkaline phosphatases were determined by automated methods as described previously (21).

*Identification and Determination of Lactoferrin.*—For electrophoretic and immunological analyses, leukocyte homogenates and fractions were frozen and thawed six times for disruption of the particles, and extracted overnight in 1 M NaCl at 4°C. Insoluble material was then removed by centrifugation. When necessary, the clear supernatants were concentrated by ultrafiltration.

An antiserum against lactoferrin from rabbit heterophil leukocytes was obtained from a goat, which was injected intramuscularly at intervals of 2 wk with an extract of about 10<sup>8</sup> rabbit heterophil leukocytes prepared as described above. 1 ml of Freund's complete adjuvant was injected together with the 2 first portions of antigen. The goat antiserum was collected 1 wk after the fourth injection, absorbed with rabbit serum, with which it showed a weak reaction, and then used as such for the determination of lactoferrin. As will be shown in the results, the antiserum obtained by this procedure showed a high degree of specificity for lactoferrin, thanks to the remarkable immunogenic properties of this protein.

Lactoferrin was identified by electrophoresis on cellulose acetate in HCl-barbiturate buffer pH 8.6 (Beckman Microzone Apparatus, Beckman Instruments, Inc., Fullerton, Calif.), by immunoelectrophoresis on cellulose acetate (11), and by immunodiffusion. Radioautography after binding of <sup>59</sup>Fe served as a specific detection technique. The marker, in the form of a trace of labeled ferric citrate with a specific activity of 12.7 mCi/mg (New England Nuclear Corp., Boston, Mass.) was added together with 1 M NaCl at the extraction step. X-ray films, Curix RP (Agfa-Gevaert, Antwerp, Belgium), were employed for radioautography.

<sup>1</sup> The B-XIV rotor was put at our disposal by Dr. N. G. Anderson, Oak Ridge National Laboratory, under subcontract No. 3081, under W-7405-Eng 26, between Union Carbide Corporation and The Rockefeller University.

The quantitative determination of lactoferrin was carried out with the goat antiserum by means of the technique of radial immunodiffusion in agarose gel described by Mancini et al. (27). The samples were extracted as described above and assayed without concentration. A leukocyte extract at five different concentrations was used as the immunodiffusion standard on each single plate. The highest concentration of the standard was represented by the extract of a suspension containing 15,000 cells/mm<sup>3</sup>.

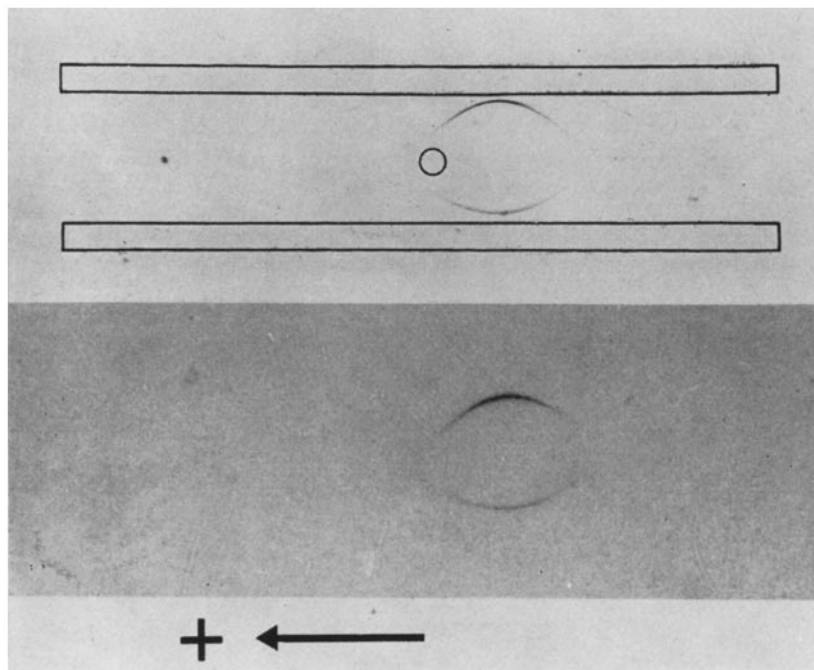


FIG. 1. Immunoelectrophoresis on cellulose acetate of rabbit leukocyte extract supplemented with <sup>59</sup>Fe, developed by the goat antiserum. The corresponding radioautography (bottom).

## RESULTS

### *Identification of Lactoferrin in Rabbit Heterophil Leukocytes*

*Immunological Identification.*—Immunoelectrophoresis of a rabbit heterophil leukocyte extract against the goat anti-rabbit leukocyte serum showed a single, sharp precipitin line. This line was detectable also by radioautography when <sup>59</sup>Fe was added to the leukocyte extract (Fig. 1).

As shown in Fig. 2, the same antiserum also gave a precipitin line with purified guinea pig lactoferrin, and the latter line partially fused with the precipitate obtained with the rabbit leukocyte extract. This cross-reaction, as

well as the demonstration of the iron-binding capacity of the rabbit leukocyte antigen, qualifies the single antigenic component recognized by the goat antiserum in the leukocyte extract as the rabbit equivalent of guinea pig lactoferrin.

The fact that this antiserum was obviously specific for rabbit lactoferrin justified its use for the immunochemical quantitation of this protein.

*Electrophoretic Identification.*—When extracts from rabbit heterophil leukocytes were fractionated by electrophoresis on cellulose acetate at pH 8.6, three cathodic bands could be distinguished. The middle band appeared as the only labeled component on X-ray plates when  $^{59}\text{Fe}$  was added to the extract. Fig. 3

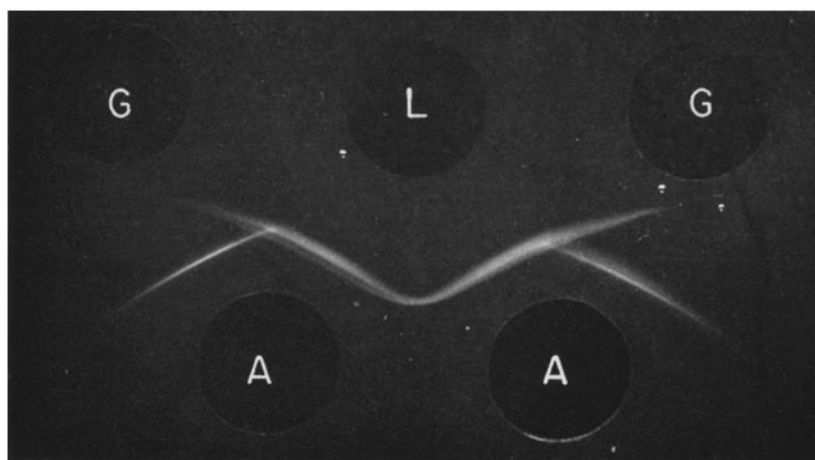


FIG. 2. Immunodiffusion plate showing the partial fusion of the precipitin lines produced by the goat antiserum (A) with purified guinea pig lactoferrin (G) and rabbit leukocyte extract (L).

shows the electrophoretic and radioautographic patterns of five  $^{59}\text{Fe}$ -labeled samples, an extract of rabbit heterophil leukocytes, rabbit serum, rabbit milk, and human and guinea pig lactoferrin. A radioactive band having about the same electrophoretic mobility as guinea pig lactoferrin is clearly detectable by radioautography in the rabbit leukocyte extract, both before and after dialysis at pH 4.0, thus providing further confirmation of the lactoferrin nature of the iron-binding protein of rabbit heterophil leukocytes.

In contrast, no radioactive band is seen in serum or milk after dialysis at pH 4.0. Before dialysis, however, the radioactive band corresponding to transferrin, which unlike lactoferrin releases its iron in weak acid medium (28), is visible in both the milk and serum samples in the  $\beta$  region. It is known that lactoferrin is practically absent in rabbit milk (29).

*Intracellular Distribution of Lactoferrin in Rabbit Heterophil Leukocytes*

*Immunological Analysis of Fractions.*—Fig. 4 shows the results of a fractionation of rabbit heterophil leukocytes carried out by zonal differential centrifugation. The distributions of alkaline phosphatase, acid *p*-nitrophenyl phosphatase, peroxidase, and protein reproduce former results obtained by this method (21).

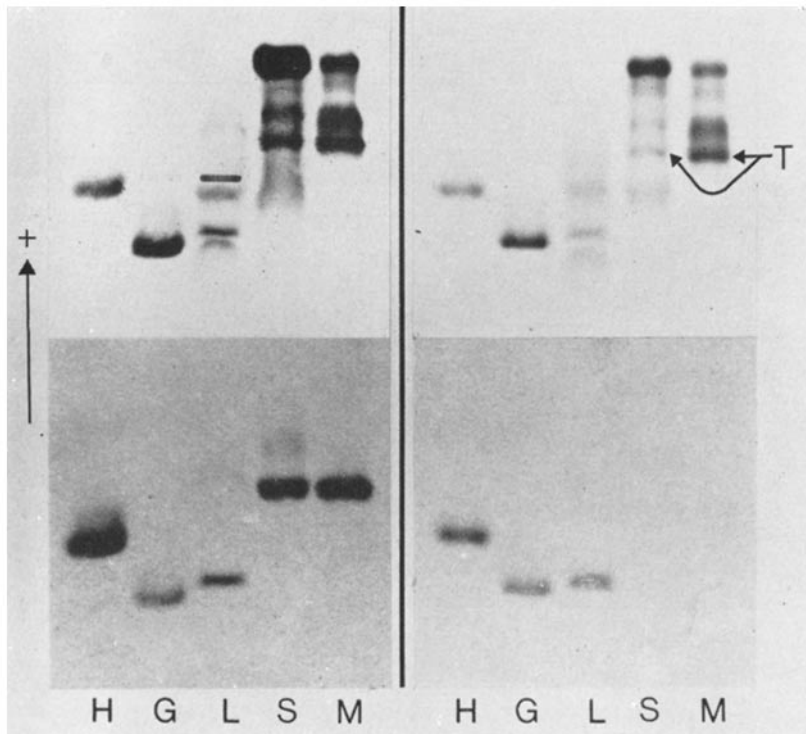


FIG. 3. *Left.* Electrophoresis on cellulose acetate (top) and the corresponding radioautography (bottom) of human (H) and guinea pig (G) lactoferrin, rabbit heterophil leukocyte extract (L), rabbit serum (S), and rabbit milk whey (M), supplemented with  $^{59}\text{Fe}$ . The milk whey was concentrated five times by ultrafiltration. *Right.* The same but with samples dialyzed against 500 volumes of 0.1 M citrate buffer pH 4.0, containing 1 M NaCl. T, transferrin.

Lactoferrin, determined in each fraction by radial immunodiffusion, shows a distribution that closely parallels that of alkaline phosphatase, thus suggesting that the iron-binding protein is localized in the specific or secondary granules. Exactly the same results were obtained in a second experiment of this type, not illustrated here.

A third fractionation was performed by isopycnic centrifugation in the Beau-

fay rotor. In this experiment, most likely because the rotor was emptied somewhat too fast, the two major bands corresponding to the two main populations of granules were wider and overlapped each other more than in earlier experiments (22, 23). Nevertheless, the density distribution histograms (Fig. 5)

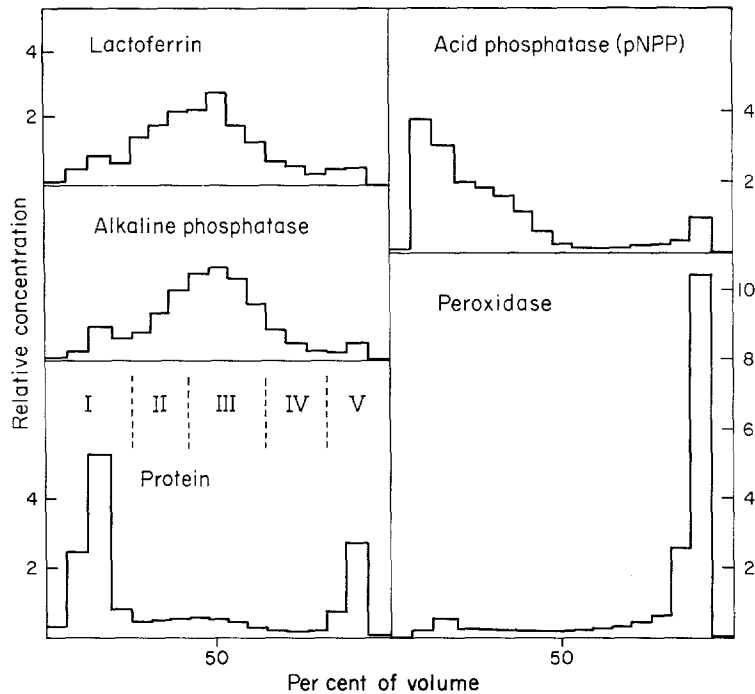


FIG. 4. Fractionation of subcellular components of rabbit heterophil leukocytes by zonal differential centrifugation at 6500 rpm for 15 min. Graphs are normalized distribution histograms as a function of the volume collected. Radial distance increases from left to right. Ordinate is concentration in fraction relative to concentration corresponding to uniform distribution throughout the gradient. Percentage recoveries were: 91 for protein, 99 for myeloperoxidase, 89 for alkaline phosphatase, 81 for acid-*p*-nitrophenyl phosphatase (pNPP), and 106 for lactoferrin.

confirm clearly the association of lactoferrin with alkaline phosphatase observed in the zonal sedimentation experiments (Fig. 4).

*Electrophoretic Analysis of Fractions.*—The fractions collected by zonal differential centrifugation were pooled as indicated in Fig. 4. The five pooled fractions were then prepared for electrophoresis and radioautography as described under Methods. Fig. 6 shows the electrophoretic and radioautographic patterns of the five pooled fractions. Radioautography reveals an iron-binding band with electrophoretic mobility corresponding to that of serum gamma

globulin in the fractions II, III, and IV. The heaviest radioautographic trace is found in fraction III, which contains most of the specific or secondary granules of the preparations, as indicated by the corresponding alkaline phosphatase activity. On the contrary, almost no radioactivity is detectable in

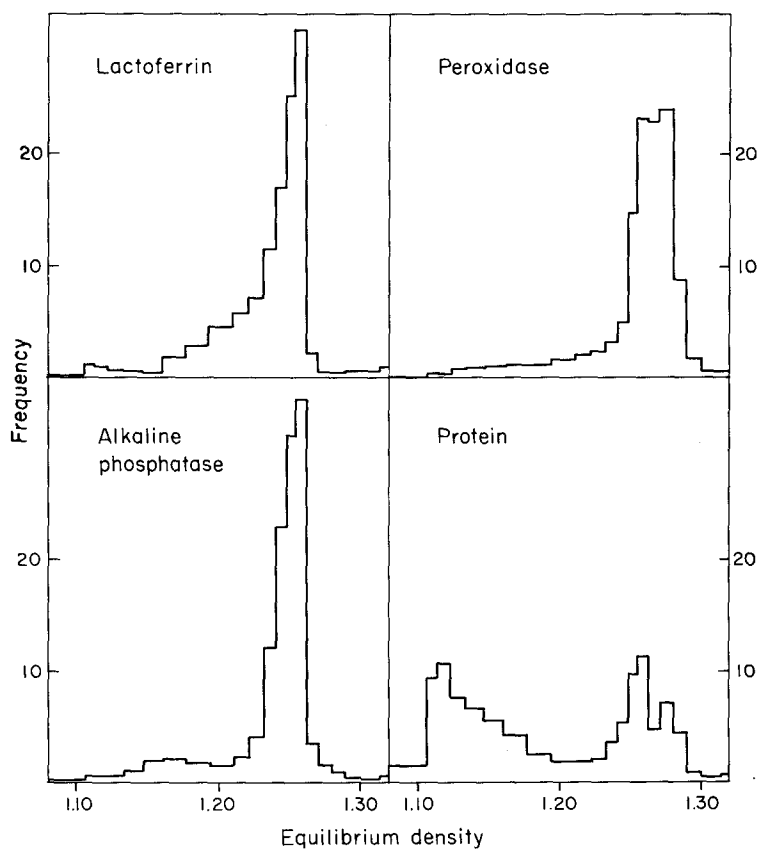


FIG. 5. Density equilibration of subcellular components of rabbit heterophil leukocytes. Graphs are frequency distribution histograms as a function of density. Percentage recoveries were: 101 for protein, 96 for myeloperoxidase, 84 for alkaline phosphatase, and 115 for lactoferrin.

fraction I, which is a heterogeneous fraction free of large granules (21), nor in fraction V, which carries the bulk of the peroxidase-containing azurophil or primary granules. These results thus confirm those obtained by quantitative immunodiffusion.

*Absorption Spectrum of Fraction III.*—During concentration, sample III became progressively pink. The absorption spectrum of the concentrated

sample showed a small shoulder in the region of 450–460 nm, corresponding to the absorption maximum of lactoferrin (7–10).

#### DISCUSSION

By immunizing a goat with a crude preparation of rabbit heterophil leukocytes, we have obtained an antiserum practically specific for a single component of the leukocyte preparation. This component binds iron and shows about the same electrophoretic mobility as does human or guinea pig lactoferrin. On the other hand, the antiserum cross-reacts strongly with purified guinea pig lactoferrin. There can be little doubt, therefore, that the antigenic

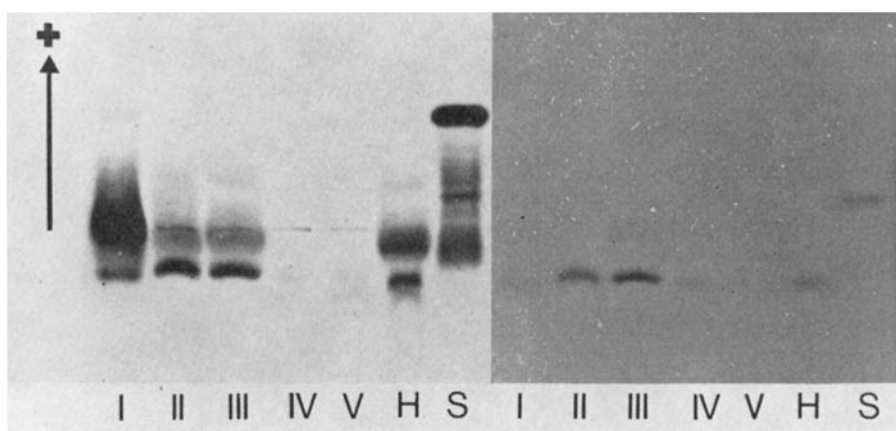


FIG. 6. *Left:* Electrophoresis on cellulose acetate of the five leukocyte fractions separated by zonal differential centrifugation (I–V), of the total leukocyte homogenate (H), and of rabbit serum (S). All samples were supplemented with  $^{59}\text{Fe}$ . *Right:* The corresponding radioautography.

component detected in this manner represents lactoferrin, a protein known to possess very strong immunogenic properties. In turn, the goat antiserum may be considered a sensitive and specific analytical tool for the detection and determination of rabbit heterophil leukocyte lactoferrin, in spite of the crude antigenic mixture used for its preparation.

The possibility that transferrin, rather than lactoferrin, may have been the antigen responsible for the observed reactions may be ruled out on several accounts. Rabbit transferrin, as we have shown here, resembles other transferrins (28) in releasing its bound iron at pH 4.0, whereas the leukocyte protein retains its iron under these conditions, as do other lactoferrins (7–10). In addition, the electrophoretic mobility of transferrin is greater than that of the iron-binding protein of leukocytes. Finally, the goat antiserum was freed of any antibody



against transferrin that might have been present, by exhaustive absorption with rabbit serum.

Our fractionation experiments demonstrate that the lactoferrin of rabbit heterophil leukocytes has an intracellular distribution almost identical to that of alkaline phosphatase. This was shown, with satisfactory recoveries, by means of quantitative immunological analysis of the fractions with the specific goat antiserum, and confirmed on purified fractions by electrophoresis and radioautography, as well as by the existence of an absorption band at 460 nm. Furthermore, the parallelism between lactoferrin and alkaline phosphatase was observed in two different types of fractionation, one depending on differences in sedimentation rate and the other on differences in equilibrium density, and both yielding fractions of great morphological homogeneity (21-23). There can be little doubt, therefore, that lactoferrin belongs to the same granules as does alkaline phosphatase. These have been identified biochemically (21-23) and cytochemically (15, 18, 20) as the specific or secondary granules. According to the electrophoretic analysis of the fractions enriched in these granules, lactoferrin appears to be one of their major protein components. The compound migrating in front of lactoferrin has been identified as alkaline phosphatase.<sup>2</sup>

The finding that lactoferrin is associated with the specific granules agrees with the observation, reported in a preceding paper (11), that lactoferrin appears in the myeloid cells of the human bone marrow only at the promyelocyte stage. This is when the specific granules are formed, whereas the azurophil granules develop at an earlier maturation stage (16, 17).

Biological sources of lactoferrin often contain lysozyme (4, 30). This parallelism holds true for the heterophil leukocytes as well, where it extends even to the subcellular level, since lysozyme is an important constituent of the specific granules that contain alkaline phosphatase and lactoferrin (21-23). However, lysozyme is not restricted to these granules, as are the other two proteins. About one-third of the lysozyme activity of rabbit heterophil leukocytes is found in the azurophil or primary granules, which contain peroxidase and a number of acid hydrolases characteristic of lysosomes (21-23).

Since lactoferrin and lysozyme occur together in a number of secretory fluids, it is tempting to assume that they may be present in the same secretion granules in the corresponding glandular cells, and that these granules bear some kind of relationship to the specific granules of the leukocytes. However, the latter appear to be normally discharged into phagocytic vacuoles, rather than outside the cells. The function of lactoferrin in the phagocytic process is obscure. It may, together with lysozyme and the bactericidal cationic proteins that are found in the leukocyte granules (24, 31), play a role in the defense against

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<sup>2</sup> Masson, P. L., and M. Baggiolini. Unpublished results.

bacterial invasion, since lactoferrin displays bacteriostatic properties, at least in media of low iron content (3, 4, 32).

#### SUMMARY

Lactoferrin has been identified in rabbit heterophil leukocytes on the basis of its immunological reactivity, electrophoretic mobility, acid-resistant iron-binding properties, and spectral characteristics.

Leukocyte lactoferrin was found to be exclusively localized in the specific (secondary) granules, which have been resolved from other subcellular components by zonal differential centrifugation and by isopycnic equilibration.

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#### BIBLIOGRAPHY

1. Masson, P. L., J. F. Heremans, and C. Dive. 1966. An iron-binding protein common to many external secretions. *Clin. Chim. Acta.* **14**:735.
2. Masson, P. L., J. F. Heremans, E. Schonne, and P. A. Crabbé. 1968. New data on lactoferrin, the iron-binding protein of secretions. *Protides Biol. Fluids Proc. Colloq. Bruges.* **16**:633.
3. Masson, P. L., J. F. Heremans, J. J. Prignot, and G. Wauters. 1966. Immunohistochemical localization and bacteriostatic properties of an iron-binding protein from bronchial mucus. *Thorax.* **21**:538.
4. Masson, P. L., and J. F. Heremans. 1966. Studies on lactoferrin, the iron-binding protein of secretions. *Protides Biol. Fluids Proc. Colloq. Bruges.* **14**:115.
5. Gordon, W. G., J. Ziegler, and J. J. Basch. 1962. Isolation of an iron-binding protein from cow's milk. *Biochim. Biophys. Acta.* **60**:410.
6. 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.
7. Montreuil, J., J. Tonnelat, and S. Mullet. 1960. Préparation et propriétés de la lactosidérophiline (lactotransferrine) du lait de femme. *Biochim. Biophys. Acta.* **45**:413.
8. Blanc, B., and H. Isliker. 1961. Isolement et caractérisation de la protéine rouge sidérophile du lait maternel: la lactotransferrine. *Bull. Soc. Chim. Biol.* **43**:929.
9. Johansson, B. 1960. Isolation of an iron-containing red protein from human milk. *Acta Chem. Scand.* **14**:510.
10. Groves, M. L. 1960. The isolation of a red protein from milk. *J. Amer. Chem. Soc.* **82**:3345.
11. Masson, P. L., J. F. Heremans, and E. Schonne. 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. Exp. Med.* **130**:643.
12. Hirsch, J. G., and Z. A. Cohn. 1960. Degranulation of polymorphonuclear leukocytes following phagocytosis of microorganisms. *J. Exp. Med.* **112**:1005.
13. Cohn, Z. A., and J. G. Hirsch. 1960. The influence of phagocytosis on the intra-

- cellular distribution of granule-associated components of polymorphonuclear leucocytes. *J. Exp. Med.* **112**:1015.
14. Zucker-Franklin, D., and J. G. Hirsch. 1964. Electron microscope studies on the degranulation of rabbit peritoneal leukocytes during phagocytosis. *J. Exp. Med.* **120**:569.
  15. Wetzel, B. K., R. G. Horn, and S. S. Spicer. 1963. Cytochemical localization of non-specific phosphatase activity in rabbit myeloid elements. *J. Histochem. Cytochem.* **11**:812.
  16. Bainton, D. F., and M. G. Farquhar. 1966. Origin of granules in polymorphonuclear leukocytes. *J. Cell Biol.* **28**:277.
  17. Wetzel, B. K., R. G. Horn, and S. S. Spicer. 1967. Fine structural studies on the development of heterophil, eosinophil and basophil granules in rabbits. *Lab. Invest.* **16**:349.
  18. Wetzel, B. K., S. S. Spicer, and R. G. Horn. 1967. Fine structural localization of acid and alkaline phosphatases in cells of rabbit blood and bone marrow. *J. Histochem. Cytochem.* **15**:311.
  19. Bainton, D. F., and M. G. Farquhar. 1968. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. I. Histochemical staining of bone marrow smears. *J. Cell Biol.* **39**:286.
  20. Bainton, D. F., and M. G. Farquhar. 1968. Difference in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron microscopy of bone marrow cells. *J. Cell Biol.* **39**:299.
  21. Baggiolini, M., J. G. Hirsch, and C. de Duve. 1969. Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. *J. Cell Biol.* **40**:529.
  22. Baggiolini, M., J. G. Hirsch, and C. de Duve. 1970. Resolution of subcellular components of rabbit heterophil leukocytes into distinct populations by zonal sedimentation and density equilibration. *In* Symposium on Membrane Function and Electron Transfer to Oxygen. Miami, Florida. In press.
  23. Baggiolini, M., J. G. Hirsch, and C. de Duve. Further biochemical and morphological studies on subcellular components in rabbit heterophil leukocytes. *J. Cell Biol.* In press.
  24. Hirsch, J. G. 1956. Phagocytin: a bacterial substance from polymorphonuclear leucocytes. *J. Exp. Med.* **103**:589.
  25. Beaufay, H. 1966. La centrifugation en gradient de densité. Thèse d'Agrégation de l'Enseignement Supérieur, Université Catholique de Louvain, Louvain, Belgium. Ceuterick, S. A. Louvain, Belgium.
  26. Leighton, F. B., B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler, and C. de Duve. 1968. The large-scale separation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with Triton WR-1339. *J. Cell Biol.* **37**:482.
  27. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry.* **2**:235.
  28. Laurell, C. B. 1947. Studies on the transportation and metabolism of iron in the body. *Acta Physiol. Scand.* **14** (Suppl. 46): 1.

29. Baker, E., D. C. Shaw, and E. H. Morgan. 1968. Isolation and characterization of rabbit serum and milk transferrins. Evidence for difference in sialic acid content only. *Biochemistry*. **7**:1371.
30. Speece, A. J. 1964. Histochemical distribution of lysozyme activity in organs of normal mice and radiation chimeras. *J. Histochem. Cytochem.* **12**:384.
31. Zeya, H. I., and J. K. Spitznagel. 1968. Arginine-rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. *J. Exp. Med.* **127**:927.
32. Oram, J. D., and B. Reiter. 1968. Inhibition of bacteria by lactoferrin and other iron-chelating agents. *Biochim. Biophys. Acta.* **170**:351.