Lactoferrin: Its Role as a Ga-67-Binding Protein in Polymorphonuclear Leukocytes

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Gallium-67 bound to lactoferrin—an iron-binding protein found in high concentration in polymorphonuclear leukocytes—has been isolated from PMNs that have previously been incubated with Ga-67 citrate. Although the cell-labeling efficiency was highly variable (0.026–10%), much of the activity that did bind to the PNMs (74.8 ± 10%) was recovered in the supernatant after sonication and centrifugation. About half (~47%) of the PMN-bound activity was retained after dialysis and was presumably bound to macromolecules in the supernatant. When this retained activity was placed on a column containing immobilized antilactoferrin antibody, almost three quarters of the activity was bound to the column. This bound activity was (36 ± 17%) of the total activity absorbed by the PMN. The addition to the antilactoferrin column of a known antigen-antibody-dissociating agent caused the dissolution of the complex. No significant activity was bound to a control column. The findings indicate that lactoferrin is a major Ga-67-binding protein present in PMNs and suggest that it may play a major role in Ga-67 localization in an abscess. These results support the contention that molecules binding ferric iron have an important effect on Ga-67 distribution in vivo.

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Lactoferrin (LF), a protein that binds two atoms of iron with high affinity, is found in many tissues and secretions in which Ga-67 is known to localize. For example, LF is present in high concentration in human milk, colonic mucosa, nasal secretions, cervical mucus, seminal fluid, and tears (*1–3*). Previous work from this laboratory has shown that Ga-67 found in human milk (*4*) and tears (*5*) is bound to LF, suggesting the formation in vivo of a LF-Ga complex. The exact mechanism of Ga-67 accumulation in abscesses is unknown, but it is thought that polymorphonuclear leukocytes (PMNs) participate (*6–8*). Recent evidence (*9*) suggests that damaged PMNs take up increased quantities of Ga-67. PMNs contain relatively high concentrations of LF (22 μg/10^6 cells) (*10*), and Ga-67 is known to bind to LF (*4,11*). These established relationships provide a potential explanation for Ga-67 sequestration in inflammatory processes. We have used affinity chromatography to demonstrate the existence of a LF(Ga-67) complex in PMNs.

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METHODS

PMN isolation. Following informed consent, human volunteers provided peripheral venous blood, from which PMNs were isolated by dextran sedimentation followed by the Ficoll/Hypaque gradient technique (*12*). Hypotonic lysis was used to remove any contaminating red blood cells. A white-cell differential count indicated that more than 90% of the cells in the final preparation were PMNs.

Cell labeling. Approximately 10^7 cells were suspended in 1 ml of normal saline containing 5 millimol glucose and 1 millimol N-2 hydroxyethylpiperazine N′-2-ethanesulfonic acid (HEPES) buffer,* pH 7.4. To assess PMN membrane integrity, the trypan-blue exclusion test was performed either just before or after radioactivity

addition. In either case the percentage of cells excluding the dye was always between 91 and 99%. The cells were incubated with 7–28 μCi of Ga-67 citrate[†] for 30 min at 22°C. Plastic test tubes were used to avoid the loss of activity on glassware. After incubation, the PMNs were washed at least three times with cold Hank's balanced salt solution containing 1 millimol HEPES as the buffering agent.

Ga-67 lactoferrin isolation. After labeling with Ga-67, the cells were usually resuspended in 10 millimol Tris buffer (pH 8.0) containing 1 *M* NaCl and were sonicated[‡] for 3 min at 50 watts power for efficient extraction of LF (*10*). The sample was placed in an ice bath and pulse-mode sonication was used to minimize heating. (Optical microscopy of the sample after sonication showed only cell debris.) The sample was then centrifuged at 30,000 *g* for 20 min at 4°C to sludge the debris. The clear supernatant was dialyzed for 3 hr against the sonication buffer at 4°C. Control experiments showed that this step equilibrated the sample fully for the impending chromatography, and also removed small molecules found in the cytosol (water-soluble portion of cytoplasm) from the sample. The sample was split into two aliquots, and one was applied to a column containing anti-LF antibody* coupled to Sepharose 4B. The other aliquot was placed on a column containing immobilized antibody to transferrin (TF).*

To learn whether the sonication altered the subcellular distribution of Ga-67—e.g., transferred Ga-67 from the surface membrane to LF and/or other proteins—a labeled suspension of PMNs was divided into a number of duplicate aliquots. Forty microliters of a LF solution (final concentration twice the concentration of LF in PNM) and 40 μl of buffer were added to a sample and a control aliquot. These aliquots were incubated for 3, 15, and 60 min at room temperature, then centrifuged and the supernatant assayed for radioactivity. There was no significant difference in supernatant radioactivity between the control and the sample containing LF.

Affinity chromatography. Affinity columns were prepared using standard methods (*13,14*). Both the anti-LF and anti-TF, coupled to Sepharose 4B, were prepared in batches. An aliquot of each batch was then poured into a glass column,[‖] 0.5 × 18 cm, yielding approximately 1.4 ml swollen gel for each antibody column. It was determined through testing (see herein) that a column prepared in this manner could be used twice. The specificity of each antibody column was checked with solutions of LF* and TF[§] labeled with Fe-59[†] and Ga-67, but containing no unbound radionuclide (*15*). When an Fe-59-labeled LF solution (400 μg) was added to the anti-LF column, about 99% of the activity was bound. Since TF is a known Ga-67-binding protein that might be present with PMNs (*16–18*), the anti-LF column was tested for cross-reactivity with a solution of Fe-59- or Ga-67-labeled TF (1320 μg). Less than 5% of the added radioactivity was bound. A similar procedure was used to test the anti-TF antibody column, and 91% of the TF (Fe-59) activity was bound (corresponding to 418 μg TF). When Fe-59-labeled LF (156 μg) was added to the column, less than 3% of the activity was retained.

Each antibody column bound approximately 400 μg of antigen, and there was little cross-reactivity. Since the exact amounts of LF and TF recovered from the PMNs were not determined, an exact correction for nonspecific binding could not be derived. However, since the TF and LF concentrations used in the cross-reactivity controls were approximately equal to or in excess of expected concentrations [220 μg of LF (*10*) and 60 ng of TF per 10^7 cells (*19*)], the maximum error in the observed value of LF-bound Ga-67 would be 5%, due to binding of the TF(Ga-67) complex to the anti-LF column, and 3% for the LF(Ga-67) complex binding to the anti-TF column.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN DIFFERENT FRACTIONS OF LF(Ga-67) ISOLATION PROCEDURE

	Mean	Range	
PMN labeling efficiency (N = 10)			
1. % of added activity	1.85	0.026–10	
2. absolute uptake (nCi/10^7 cells)	23.0	1.74–130.0	
	Percentage of initial radioactivity in:		
	PMN	Cytosol fraction	Macromolecular fraction
Isolated fractions (N = 4)			
1. sonicate supernatant (cytosol)	74.8 (59.3–87.2)*	100*	—
2. dialysis retentate (macromolecules)	47.0 (28.0–66.0)	59.4 (36.4–75.9)	100*
3. bound to anti-LF column	36.0 (17.0–57.6)	45.0 (22.0–66.0)	73.9 (61.4–87.3)
4. bound to anti-TF column	3.0 (1.9–3.5)	3.8 (2.5–4.4)	7.0 (3.9–11.7)

* Mean + range.

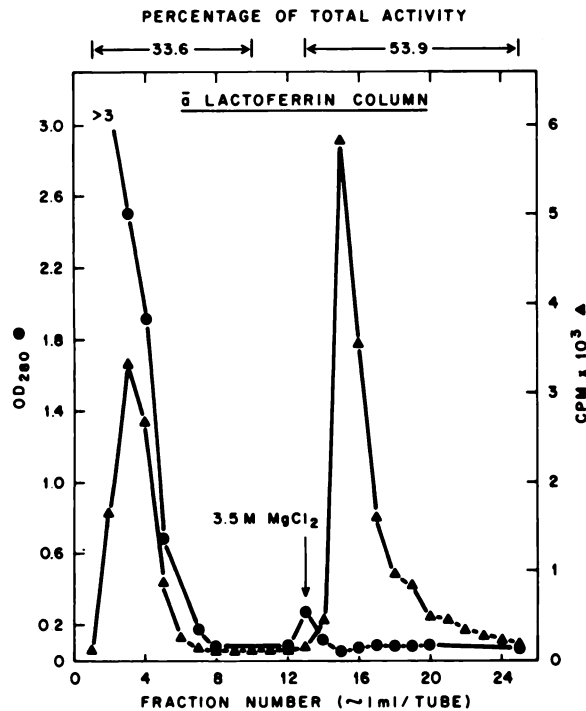

Fig. 1. Elution profile of dialyzed supernatant from PNMs incubated with Ga-67 citrate, sonicated, centrifuged (for details see Methods), and then added to column (0.5 × 18 cm) containing 2 mg of anti-lactoferrin antibody coupled to 1.4 ml wet Sepharose 4B. Starting buffer was 10 millimol Tris, pH 8.0, containing 1 *M* NaCl, and 3.5 *M* $MgCl_2$ was added at arrow. ● = protein concentration as measured by optical density at 280 nm; ▲ = radioactivity. Numbers at top of figure represent percentage of added activity in particular fractions as indicated.

RESULTS

The amounts of Ga-67 bound to the PMN were extremely variable, both in terms of percentage of the radioactivity taken up by the cells and in absolute uptake of Ga-67, nCi per 10^7 cells (see Table 1). Almost three quarters of the radioactivity bound to the PMNs was present in the supernatant of the sonicated cells. The portion of the Ga-67 activity present in the postsonication pellet was firmly bound. Repeated washes and sonications to ensure total cell disruption recovered only about 20% of the radioactivity in the postsonication pellet. The sample was dialyzed to determine the fraction of Ga-67 activity bound to macromolecules in the supernatant: it contained about half of the originally added activity (Table 1).

The dialyzed fraction of the cell sap containing the macromolecules was applied to both anti-LF and anti-TF affinity columns. Figure 1 shows the elution pattern of the anti-LF column from one such experiment. The starting buffer, 10 millimol Tris containing 1 *M* NaCl, pH 8.0, eluted ~25% of the added radioactivity, whereas almost 75% (Table 1) was apparently bound to the column. To test whether this represented a specific LF(Ga-67) complex bound to the antibody, a 3.5 *M* solution of $MgCl_2$, pH 6.4, was added, since high concentrations of $MgCl_2$ (2–5 molar) and acid solutions are known to cause the breakdown of the antigen-antibody complex (*20,21*). The $MgCl_2$ eluted a small protein fraction containing 81% of the bound activity. The $MgCl_2$ solution evidently also caused some dissociation of the LF(Ga-67) complex.

To ensure that the $MgCl_2$ solution did dissociate the LF(anti-LF) complex, a control experiment was performed. Commercially purchased LF, labeled with Fe-59, was added to an anti-LF antibody column. The elution profile is shown in Fig. 2. The starting buffer eluted only an insignificant amount of activity while some protein was eluted from this column. The eluted protein probably represented contaminants that are known to be in the commercial preparation. Upon the addition of 3.5 *M* $MgCl_2$, a protein fraction containing 82.4% of the activity originally bound to the column was eluted. The percentage of the eluted radioactivity corresponded well with the previous experiment (see before). Therefore, the $MgCl_2$ did indeed dissociate the LF(anti-LF) complex. This confirms that the protein and radioactivity peak from the previous chromatogram (Fig. 1) represented a dissociation of a specific complex and demonstrated the presence of the LF(Ga-67) in the soluble supernatant of the PMN sonicate. Moreover, this LF-bound activity constituted about three quarters of the Ga-67 bound to macromolecules present in the PMN (Table 1). When the overall recovery of Ga-67 activity was determined, based on the total cell-bound Ga-67, about 40% of this radioactivity recovered in the cells was retained by the anti-LF antibody column (Table 1). This was not corrected for losses in the pellet following sonication, or for

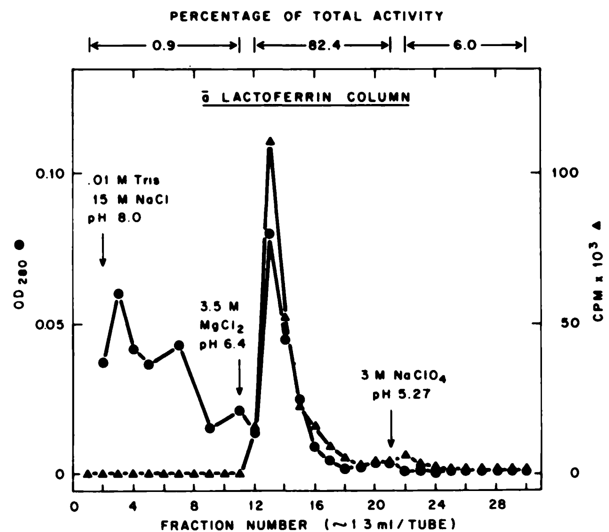

Fig. 2. Chromatogram of commercially obtained lactoferrin, labeled with Fe-59 and applied to column containing immobilized anti-lactoferrin antibody. Tris buffer (10 millimol, pH 8) containing 0.15 *M* NaCl was starting buffer; all other conditions are as described in Fig. 1.

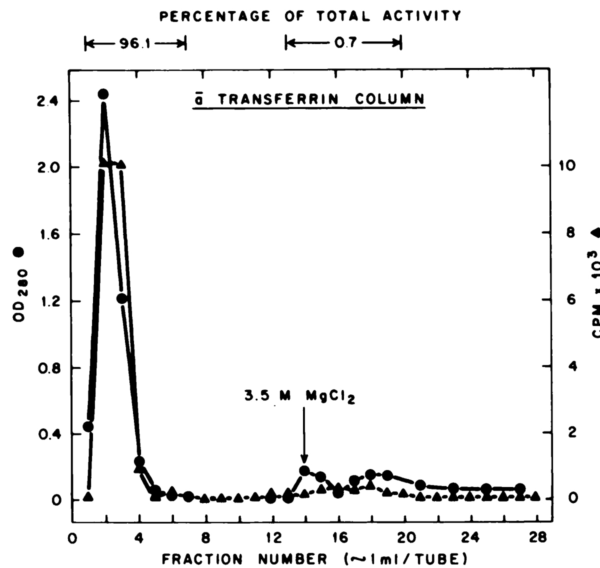

Fig. 3. Chromatogram of aliquot of sample in Fig. 1, which was added to column containing anti-transferrin antibody. Starting buffer was 10 millimol Tris, pH 8.0, containing 1 *M* NaCl; all other conditions are as described in Fig. 1.

losses due to dialysis. This figure, therefore, represents a minimum estimate for the amount of Ga-67 activity bound intracellularly to LF.

The second aliquot from the dialyzed cell sap obtained from the PMN sonicate (the macromolecular fraction) was added to a column containing immobilized anti-TF antibody. This column served to detect the presence of TF(Ga-67) and provided a control for any nonspecific binding of Ga-67 activity. The chromatogram from this column is shown in Fig. 3. Almost all the protein and activity were eluted with the starting buffer (10 millimol Tris containing 1 *M* NaCl, pH 8.0). On average, only 7.0% was bound (Table 1). The addition of the dissociating agent effected the elution of only a small quantity of protein containing an insignificant amount of radioactivity (Fig. 3). In order to ensure that this lack of binding was actually due to the absence of the TF(Ga-67) molecule rather than to the dissociation of Ga-67 from the protein due to the high ionic strength buffer, an additional experiment was performed. Conditions known to maximize the affinity of Ga-67 for TF were used (*22*). This change in conditions had no influence on the elution profile; little activity or protein was bound to the anti-TF antibody column under either condition. This indicated that Ga-67 in PMNs is not bound to TF, and there is little nonspecific Ga-67 bound to the columns.

DISCUSSION

These results indicate that PMNs, which are rich in LF (22 $\mu g/10^6$ cells), are capable of forming a LF(Ga-67) complex in vitro. Moreover, LF appears to be the major binding protein for Ga-67 in the neutrophil. These findings complement previous results, which demonstrated the presence in vivo of LF(Ga-67) complex in both milk (*4*) and tears (*5*) obtained from patients receiving Ga-67 citrate.

Our present findings also are consistent with the previously observed preference of Ga-67 to bind to neutrophils [isolated from either normal individuals (*9*) or patients with chronic granulocytic leukemia (*6*)] rather than to lymphocytes, which contain virtually no cytoplasmic LF.

The variability in labeling efficiency of Ga-67 for PMN was surprising. However, the values we observed are within the range of variability noted in the literature: 20 nCi (*8*) to 1.1 nCi (*9*) per 10 million cells. We have investigated this problem and find that the most important single factor influencing in vitro uptake is the citrate concentration in the incubation medium (Weiner, Cohen, Hoffer, et al. in preparation). However, the variation in labeling efficiency that we observed had *no* systematic influence on subcellular distribution of Ga-67: whereas variability in cell labeling was high, the portion of the cellular activity associated with LF was relatively constant (see Table 1).

We have assumed that the LF(Ga-67) complex is formed by the uptake of gallium into the cytoplasm, where the LF is present. This is somewhat in conflict with the work of Tsan et al. (*9*), who have suggested that Ga-67 binds to the PMN surface membrane and penetrates the cell only when the membrane is damaged. Our cells were assayed either before or after incubation with Ga-67 by the trypan blue test, and showed a high percentage (91–99%) with nonleaky membranes. Moreover, a control experiment (see Methods) suggested that the sonication process did not cause any artifactual transfer of membrane-bound Ga-67 to LF, and that the LF could not remove Ga-67 from labeled PMNs. Either of these transfers of Ga-67 to LF might be expected if a majority of the radioactivity were surface-bound. Thus at present it is not apparent why our data appear to show most activity in the cytoplasm and relatively little surface-bound activity, in contrast to the data of Tsan et al. (*9*).

Since the presence of a LF(Ga-67) complex both in vivo and in vitro has been demonstrated, there is a significant probability that this iron-binding macromolecule plays an important role in gallium localization in inflammatory lesions. This may be accomplished by a variety of mechanisms. First, Ga-67 may bind to LF in circulating neutrophils and subsequently be transported to inflammatory sites. It is well known that PMNs are attracted to sites of inflammation (*23*). However, our results, as well as a recent study by Tsan et al. (*9*), suggest that, in vivo, PMNs bind relatively little Ga-67. Moreover, in patients receiving Ga-67 there is little cell-bound radionuclide in the blood (*24*), suggesting that this pathway may be relatively unimportant.

Second, and probably more important, apo-lactoferrin (iron deficient) may be deposited at sites of inflammation by PMNs and secondarily bind Ga-67. Preliminary results indicate that Ga-67 has a high affinity for LF, although not as high as that of Fe(III) (*11*). A dramatic increase in LF concentration has been found in a number of inflammatory processes (*25–28*). It has been suggested that this increased concentration of LF usually results from PMNs migrating to the inflammatory site and depositing this protein (*25*). Supporting this concept, Spitznagel and his colleagues have shown that in response to a phagocytic challenge, the intact PMN releases about 45% of the LF present in the secondary granules of the cell (*29,30*). The majority of this LF (86%) is secreted into the extracellular space rather than into phagocytic vacuoles. Moreover, Wright et al. (*31*) have shown that PMN exudates bind Ga-67 avidly, and the increased binding was correlated with increased extracellular LF concentration in the exudates. Thus, LF specifically deposited by PMNs at inflammatory sites would provide the abscess exudate with a large number of potential Ga-67-binding molecules.

Finally, LF could participate in Ga-67 localization in an inflammatory process because of *de novo* synthesis at the inflammatory site. LF is formed by glandular epithelial cells (*1*) and is found in most of the major secretions that bathe mucosal surfaces (*2,3*). Considering the antimicrobial action of this protein (*32–34*), it seems likely that LF protects against local mucosal infections. Supporting this hypothesis, increased *de novo* LF synthesis has been found in a number of inflammatory diseases, namely, human pancreatitis (*26*), bovine mastitis (*27*), and human salivary disease (*28*).

The presence of an extracellular Ga-67-binding agent, LF, could explain two recently reported laboratory findings. First, when the exudates from abscesses induced in rabbits by several methods were centrifuged, the supernatants contained >70% of the total activity (*9,35*). A portion of the supernatant activity could represent a LF(Ga-67) complex, since rabbit neutrophils are known to contain LF (*36*). [Part of the activity in bacterial-induced abscesses could also represent Ga-67 bound to the bacterial iron-transport molecules, siderophores (*22,37*).]

Second, when a biopsy of an abscess induced in rats was subjected to autoradiography, the Ga-67 activity was diffusely present in the pus (*38*). The only cell-associated activity was bound to macrophages, and no significant activity was found in PMNs. This is consistent with earlier work showing that after injection, Ga-67 found in the thymus, spleen, and lymph nodes of leukemic and nonleukemic AKR mice was bound mainly to macrophages or histiocytes, with relatively little in PMNs (*39*). It has been demonstrated that LF binds to mouse macrophages and the iron present on the molecule incorporated into these cells (*40,41*). Moreover, it has been suggested that human macrophages or monocytes possess LF-binding sites (*10*). Although the presence of LF has not been demonstrated in rat PMNs (*42*), it is possible that the cell-bound activity in the rat abscess was LF(Ga-67) complexes bound to the macrophage cell surface or incorporated into the cell [LF would be present due to its release (exocytosis) from accumulated PMNs].

In summary, our present findings establish that a majority of Ga-67 bound to neutrophil homogenates is associated with LF and suggest that LF is probably the major binding protein for Ga-67 in these cells. We therefore believe that Ga-67 localization in most lesions probably results from a combination of at least two mechanisms. Ga-67 would bind to LF deposited at the site of inflammation by PMNs or other LF-synthesizing cells. Second, as has been described elsewhere (*22,37*), siderophores would foster direct bacterial uptake of Ga-67. The common denominator of this localization is the similarity of Ga-67 to the ferric ion, and the role of both siderophores and LF as molecules that bind ferric iron, and therefore gallium.

FOOTNOTES

* Calbiochem Inc.

† New England Nuclear Corp., North Billerica, MA.

‡ Heat System Ultrasonics, Inc.

‖ BioRad Laboratories, Inc.

§ Sigma Chemical Co.

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