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The Association of Ga-67 and Lactoferrin

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Activity was seen in the breasts of a patient with galactorrhea 72 hr after intravenous injection of Ga-67 citrate. Differential protein separation of breast secretion, extracted from the breast, revealed that the Ga-67 was contained primarily in the lactoferrin-rich protein fraction. Additional studies on partially purified lactoferrin revealed that lactoferrin binds Ga-67 more avidly than does transferrin. Since lactoferrin is present in high concentration not only in human colostrum and milk, but also in neutrophilic leukocytes, bone marrow, spleen, colon, tears, and in genital, salivary, and nasopharyngeal secretions, binding of Ga-67 to lactoferrin may explain the localization of Ga-67 in certain normal tissues and inflammatory lesions.

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Gallium-67 localizes in the breast tissue, colostrum, and milk of lactating women (*1–4*). Paterson and associates have suggested that an understanding of the mechanism of Ga-67 uptake in the breast and milk might provide a clue to the mechanism of Ga-67 uptake in tumors and inflammatory disease (*5*). The purpose of this study was to investigate the distribution of Ga-67 activity among the human milk proteins and to determine the affinity of Ga-67 for one of these proteins, lactoferrin.

MATERIALS AND METHODS

Bilateral breast activity was observed on a scan performed on a 40-year-old female patient 72 hr following intravenous injection of 8 mCi of Ga-67 citrate* (Fig. 1). She had been suffering from galactorrhea for 16 years. Two years ago she was diagnosed as having acute myelogenous leukemia and was being treated at the time of the scan with Daunorubicin and Cytarabine. Approximately two weeks before the scan, she developed upper back pain. The scan was performed to determine whether her back pain was due to tumor infiltrating the spinal cord. No abnormal uptake was observed in the cord region. The patient consented to having specimens of breast secretion extracted. The specimen used for this study was a 1-cc portion of a 2.5-cc extract that was obtained 10 days after injection of the Ga-67 citrate.

The 1-cc secretion specimen was defatted by refrigeration at 2°C for 24 hr, followed by centrifugation at 34,000g for about 15 min. (The Ga-67 activity of the specimen was determined before and after defatting.) The resulting clear amber fluid, containing the milk proteins, was fractioned 20 days after injection according to the method of Johanson (*6*). A summary of the method is outlined in Fig. 2. The specimen was diluted to 4 cc total volume by addition of distilled water. The casein fraction was precipitated by acidification to pH 4.6 with 1 *M* H_2SO_4, centrifugation at 34,000g for 30 min, and filtration of the supernatant through a 0.45-μ membrane filter. The filtered supernatant, containing

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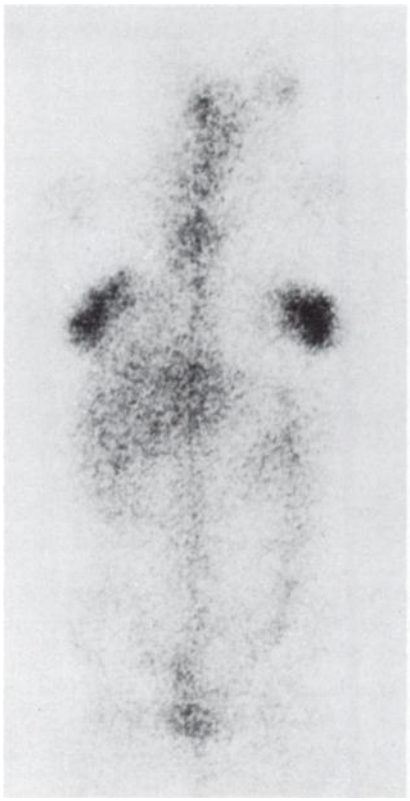

FIG. 1. Anterior view of torso 72 hr following intravenous injection of Ga-67 citrate in patient with galactorrhea of 16-year duration. Marked bilateral breast activity is present.

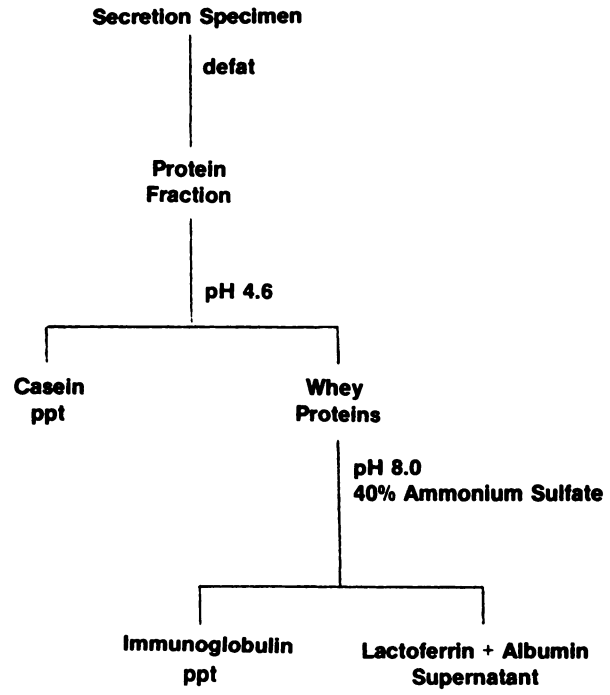

FIG. 2. Schema for separation of protein fractions from secretion specimen.

the whey proteins, was brought to pH 8 by addition of NH_4OH. The globulin fraction was precipitated by addition of ammonium sulfate to 40% saturation and separated by centrifugation at 34,000g for 30 min. The final supernatant, containing primarily lactoferrin and albumin, was not further precipitated. (The supernatant of a second specimen, processed in an identical manner, was counted and additional ammonium sulfate added to 80% saturation, the supernatant first being separated by centrifugation and then recounted to determine the amount of activity bound to protein.) All precipitates were redissolved in 1 cc of distilled water. The casein precipitate was redissolved by backwashing the 1-cc distilled water through the membrane filter before mixing with the protein plug resulting from centrifugation. All protein fractions were counted for 5 min for Ga-67 activity in a well scintillation counter with a 70–300 keV window.

The protein content of the acid-precipitated casein fraction was determined by the turbidimetric method of Henry et al. (*7*). The protein contents of the globulin and lactoferrin-rich albumin fractions were determined by a modified biuret method (*8*) adapted to a centrifugal analyzer.† Protein electrophoresis was performed on agarose gel (barbiturate buffer, pH 8.6, 90 V, 45 min) using 1 μl of each fraction (*9*). Immunoelectrophoresis of each protein fraction was performed in agarose gel according to Grabar and Brotin (*10*). After electrophoresis, each fraction was incubated with antibodies for human lactoferrin,‡ human transferrin,‡ and human whole serum.‡ After 42 hr of incubation at room temperature, the precipitin bands were made visible by staining with amido black. Relative content of lactoferrin and transferrin in the three protein fractions was determined by visual comparison of the density of the precipitin bands, taking the transferrin content of the final supernatant as unity.

The relative Ga-67 binding affinity was determined for a commercially available, partially purified, human lactoferrin preparation.|| Approximately 2 μCi of carrier-free Ga-67 citrate was incubated with 10 mg of the lactoferrin preparation dissolved in 5 cc of distilled water brought to pH 7.5 by addition of Na_2CO_3. Incubation was carried out at room temperature for 16 hr. The Ga-67 lactoferrin preparation was electrophoresed and incubated against antilactoferrin, antitransferrin, and antiserum (human) antibodies in the following manner.

Two-microliter aliquots were applied to a well on a 1% agarose film. Electrophoresis was performed for 50 min at 90 V using 0.05 *M* barbital buffer (pH 8.6) containing 0.555g calcium lactate per liter. After the completion of electrophoresis, 40 μl of the

appropriate antiserum were applied to the troughs. (For blank and control purposes, one specimen was not treated with any antiserum.) The films were incubated in a moist chamber at room temperature for 65 hr. At the end of this period, one film was submerged in 200 ml 0.9% NaCl, which was gently stirred with a magnetic bar. The second film was not washed and served as a control. The saline was replaced six times every hour. The films were then dried under an infrared heat lamp. Each film was then cut into strips that included the complete length of the gel containing the antigen and the antibody-precipitin bands. Each strip was cut into three portions and placed in a well type gamma counter, counting threshold 10 keV, and counted for ten minutes in eight repeated cycles.

Relative Ga-67 affinity for transferrin and lactoferrin was determined by competitive binding with a cation-exchange resin over the pH range 2.5–9.5. Partially purified human lactoferrin and human apotransferrin¶ were used. Both proteins were separately dissolved in 34 cc of distilled water to concentrations of 1 mg per cc. The apotransferrin was brought to about 25% iron saturation by addition of 52.2 μg of $FeCl_3 \cdot 6H_2O$. Since iron was neither added to nor removed from lactoferrin during commercial preparation, no additional iron was added to this protein solution. (The lactoferrin solution had a faint pinkish hue, indicating the presence of some iron.) Three μl of a 2 mCi/ml preparation of carrier-free Ga-67 citrate were added to each protein solution and they were then incubated for one hour at 22°C. A control solution was also prepared containing Ga-67 citrate in 34 cc distilled water. The solutions were separated into multiple 4-cc aliquots, to each of which 2 cc of AG 50W-X8 cation exchange resin, sodium form** was added. These aliquots were adjusted to a pH between 2.5 and 9.5 in one-unit increments by addition of either HCl or Na_2CO_3. The aliquots were shaken for 2 min and then incubated at 37°C for 25 min. The protein was separated from the resin by passage through a 0.45-μ membrane filter under pressure. Relative protein binding of Ga-67 was determined by counting the aliquot samples before mixing with the exchange resin and after filtration.

RESULTS

The activity in the 1-cc secretion specimen was equivalent to 1 μCi of Ga-67 on the day of injection. No significant loss of activity resulted from defatting. About 90% of the Ga-67 activity in the breast secretion was present in the lactoferrin-rich final supernatant. This fraction contained only 40% of the total protein. The remainder of the activity was divided about equally between the precipitated casein and immunoglobulin fractions. Since these fractions were not washed before counting, it is unknown whether the small amount of activity in each was actually bound to the protein precipitate or present in the small amount of supernatant trapped in the precipitate plug. All of the activity in the final supernatant fraction of the second specimen was present in the precipitate following addition of $(NH_4)_2SO_4$ to 80% saturation, indicating that all of the Ga-67 was protein-bound.

Immunoelectrophoresis showed that the final supernatant fraction contained the largest amount of lactoferrin, about ten times the sum of both the other fractions. Considerable albumin, and a small amount of transferrin, were also present in this fraction. No definite transferrin band was identified in any of the other fractions. These findings are summarized in Table 1.

On immunoelectrophoresis, the partially purified lactoferrin showed cross-reactivity with antibodies against human lactoferrin and human serum (nonspecific), but there was no cross-reactivity (or at most a trace) to transferrin. The only detectable Ga-67 activity, however, was in the lactoferrin–antilactoferrin precipitant band.

The relative affinity of Ga-67 for lactoferrin and transferrin at various hydrogen-ion concentrations is

TABLE 1. Ga-67 DISTRIBUTION, ABSOLUTE PROTEIN CONTENT, AND RELATIVE DISTRIBUTION OF TRANSFERRIN AND LACTOFERRIN IN BREAST SECRETION 10 DAYS AFTER INTRAVENOUS INJECTION OF 8 mCi Ga-67 CITRATE

	Total protein (mgm)	Ga-67 activity in cts/min ± SD	Relative transferrin content	Relative lactoferrin content
Acid ppt (pH (4.6) casein fraction	1.1	71 ± 11	Slight or absent	1
40% $(NH_4)_2SO_4$ ppt immunoglobulin fraction	12.6	80 ± 11	Slight or absent	2
Final supernatant lactoferrin and albumin fraction	8.4	1266 ± 19	1	25

shown in Fig. 3. Binding of Ga-67 to lactoferrin in this competitive-binding system remains above 40% until pH 3.5. Transferrin binding of Ga-67 decreases rapidly with increasing acidity, drops below 40% around pH 6.5, and is lower than that of lactoferrin (50% versus 77%) even in the physiologic range (pH 7.5). The control sample revealed progressive decrease in resin binding of Ga-67 at pH values above 7.5. This is presumably due to preferential citrate binding at alkaline pH.

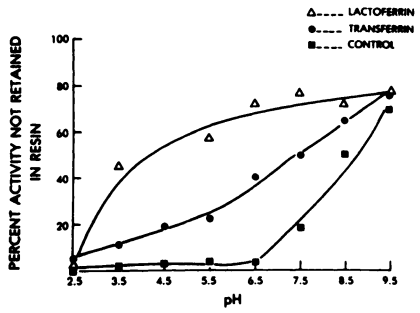

FIG. 3. Activity bound to protein vs pH, following incubation with AG-50W-X8 cation-exchange resin, sodium form, for 1 hr at 37°C. Control is Ga-67 citrate with no protein added. Lactoferrin flocculates at pH 4.5, preventing an accurate dissociation value from being obtained at that hydrogen-ion concentration.

DISCUSSION

Many investigators have observed the remarkable avidity of mammary tissue for Ga-67 citrate during lactation in humans (*1–5*). Paterson and co-workers studied the protein distribution of Ga-67 in dog's milk but could identify no specific binding protein (*5*). Tyndell et al., working with rabbit's milk, were also unable to identify a specific Ga-67-binding protein, but did find the activity confined to proteins of lower molecular weight in the 25,000 to 35,000 range (*3*).

Colostrum and milk of various species differ considerably in their content and distribution of proteins (*11*). Human milk has an unusually high concentration of lactoferrin, which accounts for about 15% of the total protein content (*12*). Lactoferrin is present in lower concentration in cow's milk but is virtually absent from the milk of rabbits and dogs (*11*).

The protein was first described in 1939 by Sorensen and Sorensen (*13*). It was subsequently isolated from cow's milk by Groves (*14*). Lactoferrin has a molecular weight of 85,000 to 90,000 and a general structure somewhat similar to that of transferrin, but it is immunologically distinct (*15*). Both transferrin and lactoferrin bind iron but lactoferrin has the greater binding affinity (*14*). Iron bound to transferrin will translocate and become bound to lactoferrin if the latter is present (*16*).

Lactoferrin is present not only in human colostrum and milk, but also in colonic mucosa, bone marrow, spleen, saliva, nasal secretions, genital secretions, and tears (*17,18*). It is a major component of the soluble-protein fraction of neutrophilic leukocytes (*19*). Virtually all of these tissues and secretions also localize Ga-67 to some extent (*20*).

The small size of the specimen we used, and its relatively low activity at the time of separation and counting, prevented immunologic confirmation that the Ga-67 activity was restricted to lactoferrin in the final supernatant. However, by incubating Ga-67 with partially purified lactoferrin derived from human milk and containing some serum proteins, we have demonstrated definite binding of the Ga-67 to lactoferrin and no binding to the serum proteins. Furthermore, we have shown that Ga-67 binds more avidly to lactoferrin than to transferrin. This preferential avidity occurs at physiologic pH and is even more pronounced in acid solutions.

When Ga-67 citrate is injected intravenously, it is at least partially bound to transferrin (*21–25*). Winchell proposes that transferrin and perhaps other proteins carry Ga-67 into proximity with tissue proteins that bind the Ga-67 more avidly and thus remove it from the carrier (*26*). Hayes and Carlton report Ga-67 binding to two proteins, with molecular weights of about 100,000 and 50,000, in rat tumor and liver (*27*). They do not speculate on the nature of these proteins. Clausen et al. has demonstrated that about one-third of Ga-67 activity in tumors is bound to ferritin while the remainder is associated with proteins of lower molecular weight (*28*). Aulbert and associates find Ga-67 in tumors associated with a protein having a molecular weight of 85,000 to 90,000, which they assume consists of transferrin and its degradation fragments (*29*).

We postulate that Ga-67 localizes in breast tissue of lactating patients because of its strong affinity for lactoferrin. The Ga-67 may be carried to the breast weakly bound to transferrin and translocate in the manner proposed by Winchell. This mechanism could also explain localization of Ga-67 in other tissues with high lactoferrin content such as neutrophilic leukocytes. Moreover, the mechanism is not necessarily restricted to Ga-67 and transferrin but serves to explain localization of any metal that binds more firmly to lactoferrin than to the carrier protein or agent. Although lactoferrin content may be elevated in some tumors (*30*), there is no detailed information on the content of this protein in most tumors and in normal liver. The presence of ferritin in liver and some tumors could provide an alternate route of binding. Even in these tissues, however, a

possible role for lactoferrin in Ga-67 binding cannot be excluded.

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FOOTNOTES

* New England Nuclear, N. Billerica, Mass.
† Centrifichem, Union Carbide Corp., New York, N.Y.
‡ Behring Diagnostics, Somerville, N.J.
|| Calbiochem, San Diego, Calif.
¶ Sigma Chemical Company, St. Louis, Mo.
** Bio Rad Labs., Richmond, Calif.

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