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Validation of Radioimmunoassay for Human Lactalbumin in the Serum by Testing the Endogenous Antibodies Interference*

P. F. ZANGERLE†‡ and P. FRANCHIMONT§

†*Centre Hospitalier de Sainte Ode, 6970 Baconfoy, Tenneville, Belgium and* §*Radioimmunoassay Laboratory, Tour de Pathologie, C.H.U. Bat. B 23, 4000 Sart Tilman par Liège 1, Belgium*

Abstract—*For re-establishing the value of human lactalbumin as a functional marker of normal and pathological activity of the breast a sensitive and specific radioimmunoassay was established with the prior important control of the interference of endogenous antibodies. The specificity of the assay was assessed by the absence of interference from other proteins in milk or in breast cyst fluid, various hormones and tumor markers. Bovine lactalbumin showed incomplete and weak cross-reactivity. By an enzymoimmunoassay method it was shown that all the 222 human sera studied contain IgG immunoglobulins which bind bovine and human lactalbumin with greater reactivity of children's serum and without relationship to the blood groups. The maximum affinity constant of these endogenous immunoglobulins determined by the radioimmunoassay method is 4.5 times greater for bovine ($K_d = 18 \times 4^{-11}$ M) than for human ($K_d = 4 \times 10^{-11}$ M) lactalbumin. These endogenous anti-lactalbumin immunoglobulins caused no interference in the radioimmunoassay as shown by the complete correlation between the concentrations of human lactalbumin previously incubated and added to sera containing high-affinity antibodies and those measured directly in the radioimmunoassay. This lack of interference was explained by the higher (22-fold) affinity constant of the rabbit antiserum against human lactalbumin ($K_d = 9 \times 10^{-12}$ M). The study of endogenous antibodies by the two enzymes and radioimmunoassay methods is needed before assessing and using a radioimmunoassay of human lactalbumin in serum.*

INTRODUCTION

LACTALBUMIN is a major protein in human milk and has a molecular weight of 14,600 [1, 2]. It is produced by the acinar cells of the mammary gland [3–5] and appears in blood during pregnancy and lactation [6, 7]. In addition to these two physiological situations, breast cells contain lactalbumin [8–14], which is secreted into the blood in some women during the menstrual cycle [7]. This milk protein has been identified in breast cancer cells by immunocytochemistry [4, 5, 9] and in the serum of women with and without breast cancer [7, 8, 10–14].

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‡To whom requests for reprints should be addressed.

Before defining the real significance of this milk protein we established a specific radioimmunoassay for human serum lactalbumin. For this purpose we studied the presence in human serum of IgG antibodies able to react with human and bovine lactalbumin [12, 15] and to interfere in the radioimmunoassay.

In this paper we have studied these immunoglobulins primarily in order to validate the radioimmunoassay, with the secondary aim of investigating their origin and their physiopathological function.

MATERIALS AND METHODS

Radioimmunoassay

Purification of human lactalbumin. Human lactalbumin was purified from human milk using

the method of Schultz and Ebner [14]. The steps included centrifugation at 100,000 *g* to eliminate fat globules and casein micelles, gel filtration on Sephadex G100 and chromatography on DEAE cellulose using gradient elution. In the latter step pure human lactalbumin was eluted by 105 mM KCl. The purity of the protein was established by its elution in a single peak after chromatography on Sephadex G100, by its migration as a single band on 1% SDS-polyacrylamide gel electrophoresis, by a single precipitation line in immunoelectrophoresis in the alpha-globulin zone in the presence of an anti-milk protein antiserum (Nordic Immunological Laboratories, Tilburg, Netherlands; RAHu/TM) and by lack of reactivity with an anti-human serum protein antiserum (Nordic Immunological Laboratories, Tilburg, Netherlands; RAHu/ielfo).

Antiserum. Rabbit antisera were raised using the method of Vaitukaitis *et al.* [16]. Fifty micrograms of purified lactalbumin diluted in 1 ml of saline was emulsified in an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) before immunizing two rabbits. Each rabbit was given 20–50 intradermal injections of 0.1 ml of the emulsion along the vertebral column. Three boosters were given by intramuscular injection of 100 μg of lactalbumin without adjuvant at 4-week intervals. The antiserum selected for radioimmunoassay had a population of antibodies with an affinity constant, measured by the method of Scatchard [17], of $K_d = 9 \times 10^{-12}$ M.

Tracer. Lactalbumin was labelled using the original method of Greenwood *et al.* [18]. The iodination mixture was immediately placed on a column of Sephadex G50 (30 × 1 cm) in order to separate labelled iodide from labelled lactalbumin. The first peak, which was the labelled protein, was placed on a column of Sephadex G75 (35 × 1 cm). Maximal radioactivity was eluted at 17, 18 and 19 ml. Binding of radioactivity in these fractions to the rabbit antilactalbumin antiserum at a dilution of 10^{-4} was 85%, while non-specific binding in the absence of the antiserum was less than 5%.

The mean specific activity calculated by the method of self-displacement [19] was 121 μCi/μg ± 12, which is equivalent to 1 atom ^{125}I per molecular of lactalbumin.

Incubation. The incubation medium consisted of 140 pg of labelled lactalbumin (± 20,000 cpm) in 0.2 ml of buffer, 0.1 ml of antiserum diluted to 2.5×10^{-5} and 0.1 ml of buffer containing from 0 to 50 ng of unlabelled lactalbumin, 0.1 ml of serum or of other biological fluids or progressively increasing amounts of organ extracts. Sorensen phosphate buffer, pH 7.5, 10 mM containing 0.2 g/l of gelatin was used for these incubations.

The first system was incubated at 4°C for 4 days; 0.1 ml of rabbit serum, 1:200 and 0.1 ml of anti-rabbit gamma globulin serum, 1:30 were added to the incubation medium. Forty-eight hours later the precipitate was separated by centrifugation, washed twice with buffer and counted.

Specificity. Possible cross-reactivity between the purified human lactalbumin and the following substances was sought:

(i) Milk protein: casein and various fragments such as kappa-casein, lactoferrin (Sigman, St. Louis, MO, U.S.A.; No. L 4881) and 'gross cystic disease fluid protein' (GCDFP 15.000) [20]; human immunoglobulin (Sigma, St. Louis, MO, U.S.A.; No. 1.3755); and human albumin (Behringer, Marburg, F.R.G.; No. ORH A 20,21). All these substances were studied at increasing concentrations up to 4 μg/tube.

(ii) Cow's milk and bovine lactalbumin (Sigma Chemical Company, St. Louis, MO, U.S.A.; No. L. 4379) to 100 ng/tube.

(iii) Other substances: purified carcinoembryonic antigen [21]; human chorionic gonadotrophin and its subunit (Serono Laboratories, Italy); alpha foetoprotein [22]; human prolactin (Calbiochem-Behring Company, San Diego, CA, U.S.A.); and hen egg-white lysozyme (Sigma Chemical Company, St. Louis, MO, U.S.A.; No. L. 6876). All of these substances were studied at increasing concentrations up to 1 μg/tube.

The effect of serum proteins was also studied, by setting up reference curves in the presence of 0.1 ml of either a pool of boys' serum or of castrated ram serum.

Detection and study of endogenous immunoglobulins which bind human lactalbumin

Method for detecting and identifying anti-lactalbumin antibodies. The investigation of endogenous immunoglobulins which react with lactalbumin was undertaken using an enzymo-immunological method in 100 sera of men, 100 sera of women and 22 sera of children (from 1 month to 2 yr old) [23, 24]. The method is based on the binding of human or bovine lactalbumin to the walls of a tube, and subsequent incubation with serum containing the anti-lactalbumin immunoglobulins. Gamma globulin binding to lactalbumin fixed to the walls of the tubes is demonstrated by using lactoperoxidase-labelled anti-gamma globulin. The human lactalbumin used was the purified protein, the bovine lactalbumin was from Sigma.

Binding of lactalbumin to the walls of the tube. Aliquots (200 μl) of sodium bicarbonate buffer,

100 mM, pH 9.4, containing 10 μg/ml of human or bovine lactalbumin were incubated in wells within polystyrene trays (Dynatech Microelisa) overnight at 4°C. After the solution was aspirated the wells were washed three times with a solution of Sorensen phosphate buffer, 50 mM, pH 7.5, containing 0.15 M sodium chloride and Tween 0.1%.

Incubation. Of a solution of Sorensen phosphate buffer, 50 mM with 150 mM of sodium chloride, 0.1% Tween and 0.5% bovine serum albumin (BSA) 150 μl, together with 50 μl of the serum to be tested or 50 μl of buffer (control), were added to the wells and incubated overnight at 4°C in a moist chamber. Human sera were used at various dilutions. Furthermore, sera were studied after preincubation for 6 days at 4°C with increasing quantities of unlabelled lactalbumin, either bovine or human, in amounts of 0, 1, 10 and 100 μg.

Demonstration of gamma-globulin bound to human or bovine lactalbumin. The incubation media were discarded and the wells were washed three times with the same buffer. Of one of the following antisera 200 μl were then added: rabbit anti-human immunoglobulin antiserum conjugated to peroxidase (Dako-immunoglobulins Copenhagen, F, Denmark, ref. P. 212); a specific rabbit anti-IgM antiserum labelled with peroxidase (Dako, ref. 215); a specific rabbit anti-immunoglobulin G labelled with peroxidase (Dako, ref. 214); or a specific rabbit anti-IgE labelled with peroxidase (Dako, ref. 238).

The solutions were incubated for 2 hr at room temperature. The wells were emptied by aspiration and washed three times using the same buffer as previously.

Two hundred microliters of a solution of 2.6 mg% $Na_2HPO_4®2H_2O$, 0.882 g% of citric acid, 75 mg% of ABTS (2,2′-azino-di(3-ethyl-ethyl-benzthiazoline)sulfonate) (Boerhinger-Mannheim, F.R.G., ref. 102.946) and 30 μl% of H_2O_2 was added to each well. The color reaction was stopped by the addition of a solution of NaOH, 100 mM, and NaN^3, 1 mM. The optical density was measured spectrophotometrically at 405 nm.

Method for calculating the affinity constant of the human immunoglobulins for human and bovine lactalbumin. The sera were selected from 222 on the basis of their significant reactivity (optical density greater than 1.2) with human lactalbumin fixed to the walls of the tubes. Of each of the sera 0.1 ml was incubated for 4 days at 4°C with 0.3 ml of incubation buffer containing progressively increasing quantities of human or bovine lactalbumin (0.6, 3.4, 6.8, 13.6, 34.2, 68.0×10^{-13} M) and 50,000 cpm of labelled human or bovine lactalbumin. Separation of the tracer endogenous immunoglobulin G complex was effected by binding to protein A-Sepharose [25].

Study of the interference of endogenous immunoglobulins in the radioimmunoassay. Two of the sera which contained a large amount of anti-lactalbumin antibody of high affinity and a small amount of low affinity respectively as determined by the previous method were incubated with 2×10^6 cpm/ml of ^{125}I human lactalbumin. After 24 hr of incubation at room temperature the mixture was chromatographed on a column of Aca 34 (100×1.5 cm). The radioactivity of each eluted fraction was measured. Then 20,000 cpm from each of these fractions were incubated for 4 days at 4°C with 0.1 ml of rabbit anti-lactalbumin diluted to 2.5×10^{-5}. At the end of this incubation the system precipitating labelled lactalbumin-antibody complexes was added for 2 days at 4°C. Binding of radioactivity to antibody was determined for each of these fractions.

Ten sera selected by the previous method and containing anti-lactalbumin immunoglobulins were incubated with 0.1, 0.2, 3, 9 and 7.8 ng of unlabelled human lactalbumin/ml. After 5 days of incubation at 4°C a radioimmunoassay of lactalbumin was carried out on each sample. The correlation between the amounts added and the amounts measured was examined.

RESULTS

Radioimmunoassay

Figure 1 shows a standard curve. The coefficient of variation of each point on the curve was less than 2%. The sensitivity defined as the smallest amount of unlabelled lactalbumin able to produce a significant displacement of the radioactivity bound to the antibody in the absence of unlabelled lactalbumin (B_0) was of the order of 8 pg/tube.

Antibody-bound labelled human lactalbumin was not displaced by milk protein such as casein, kappa-casein, lactoferrin, GCDFP 15,000, human albumin, immunoglobulin A, purified tumor markers such as CEA, alpha-fetoprotein, HCG or its alpha- and beta-subunits, prolactin, egg-white lyzozyme or bovine milk. Sera from women who were pregnant, lactating or outside these two physiological states displaced labelled lactalbumin from the antibody in a manner parallel to the standard curve (Fig. 2).

Bovine lactalbumin caused only weak displacement of labelled human lactalbumin from its antibody. The maximum displacement was 28% at the highest doses. For a displacement of 10% the immunoreactivity of bovine lactalbumin was 230 times less than that of human lactalbumin.

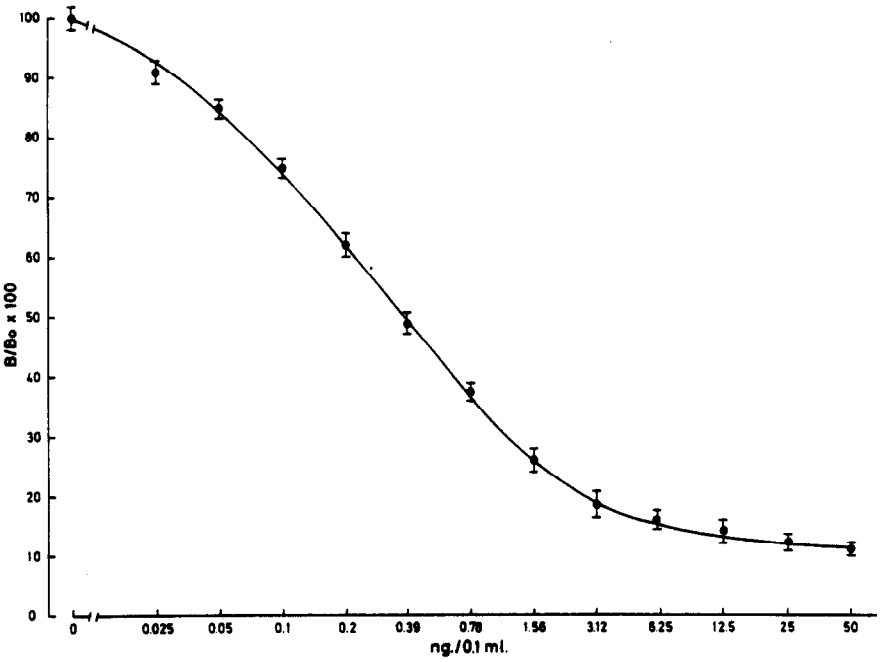

Fig. 1. Standard curve for the human lactalbumin radioimmunoassay. Ordinate: ratio of the percentage of radioactivity bound to antibody in the presence of unlabelled lactalbumin (B_x) *and that bound to antibody in the absence of unlabelled lactalbumin* (B_0). *Abscissa: amount of unlabelled lactalbumin added to the incubation medium* (ng/tube). *Each point represent the mean* ± *S.D.*

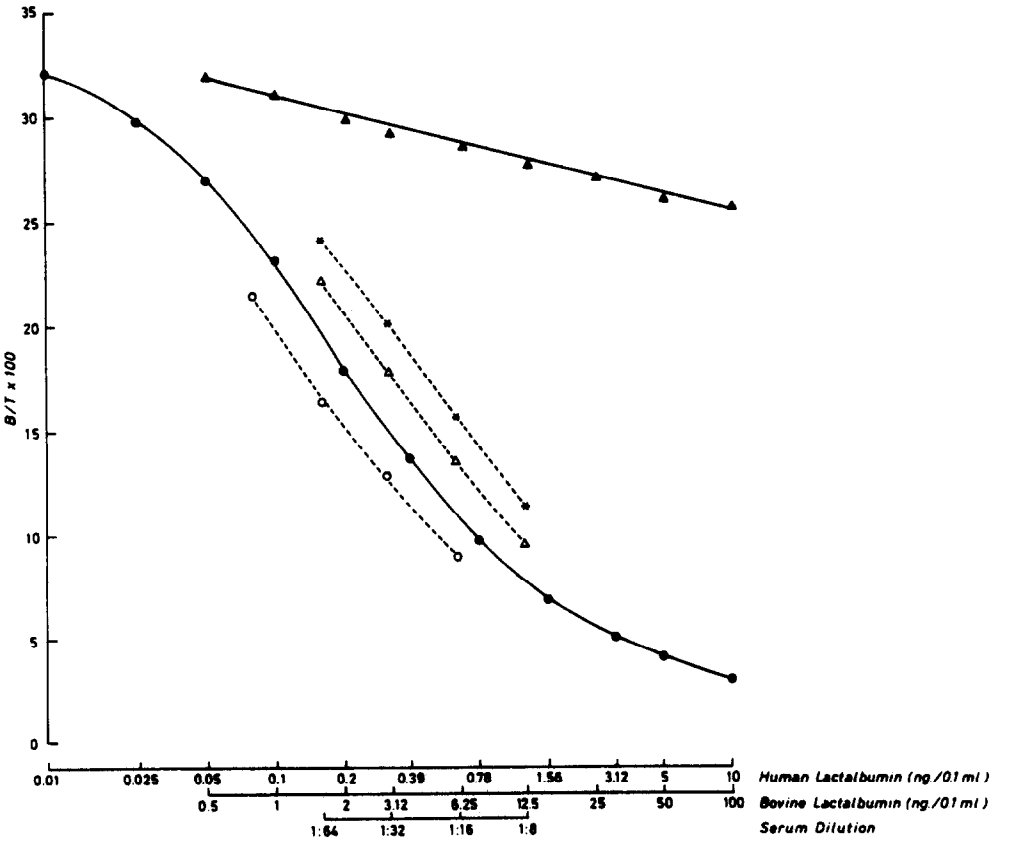

Fig. 2. Displacement of labelled human lactalbumin bound to rabbit antibody by unlabelled human lactalbumin (●——●), *by bovine lactalbumin* (▲——▲) *and by increasing dilutions of the sera of normal women* (*-----*), *pregnant women* (O-----O) *and lactating women* (Δ-----Δ). *Ordinate: ratio of radioactivity bound to antibody* (B_x) *and total radioactivity* (T). *Abscissa: amount of antigen incubated* (ng/tube). *Dilution of sera incubated.*

The curves obtained in the presence or absence of 0.1 ml of children's serum of 0.1 ml of ram serum were superimposable on the curve in buffer.

Study of endogenous immunoglobulins reacting with the lactalbumin

Detection of human anti-lactalbumin antibodies. Almost all of the 222 human sera examined reacted with human and bovine lactalbumin bound to the walls of the wells and visualized with anti-human gamma globulin antibodies labelled with peroxidase. The optical density was distinct from that seen when the wells were coated with human albumin. The sera from children had greater reactivity (Fig. 3). No difference was observed between sera of blood groups A, A-B and O.

Using antibodies directed specifically against immunoglobulin IgG, IgE and IgM labelled with peroxidase, enzyme activity was shown only with specific anti-human gamma globulin G.

Affinity constant of the human anti-lactalbumin antibodies. The binding ($B/T \times 100$) of ^{125}I bovine lactalbumin and ^{125}I human lactalbumin to the immunoglobulins of the sera selected by the first method was 64 ± 6 (S.D.) and $43 \pm 5\%$. Labelled human lactalbumin was displaced to a greater degree by bovine lactalbumin (50% displacement: 6 ± 12 ng) than by human lactalbumin (50% displacement: 50 ± 29 ng). Human lactalbumin did not displace ^{125}I bovine lactalbumin bound to antibody whilst bovine lactalbumin caused complete displacement. The affinity constant for the endogenous antibodies in serum was $K_d = 4 \times 10^{-9}$-4×10^{-11}M for human lactalbumin and $K_d = 10^{-10}$-18×10^{-11} M for bovine lactalbumin (Fig. 4).

Interference of endogenous immunoglobulins in the radioimmunoassay

When 1 ml of serum containing substantial quantities of antibody of high affinity was incubated with labelled lactalbumin and chromatographed on a column of Aca 34, the elution of radioactivity was maximal in the fractions corresponding to molecules of molecular weight 150,000. Measures of 20,000 cpm of these eluted fractions of high molecular weight had the same immunoreactivity as the fraction eluted in the 15,000 molecular weight region in buffer or in serum containing little anti-lactalbumin antibody of low affinity (Fig. 5).

When human lactalbumin was first incubated with sera containing endogenous anti-lactalbumin immunoglobulins, the amounts measured were in agreement with the amounts added whatever the affinity constant, as shown in Fig. 6.

DISCUSSION

Human lactalbumin is of potential interest as an index of mammary gland function [6, 7]. In order to re-evaluate the physiopathologic significance of this protein in the serum, a radioimmunoassay was established.

Human lactalbumin was purified using the method of Schultz and Ebner [14].

The specificity of the human lactalbumin assay was validated: with our anti-human lactalbumin serum there was no cross-reaction between lactalbumin and other milk proteins; lactoferrin, casein, human lactalbumin, immunoglobulin A; with proteins from mammary cyst fluid: GCDFP 15,000; or with cancer antigens such as CEA, AFP, HCG, HCG-alpha and -beta, egg white lysozyme and human prolactin. Bovine lactalbumin showed weak and incomplete cross-reactivity with human lactalbumin. Large amounts of bovine lactalbumin produced a

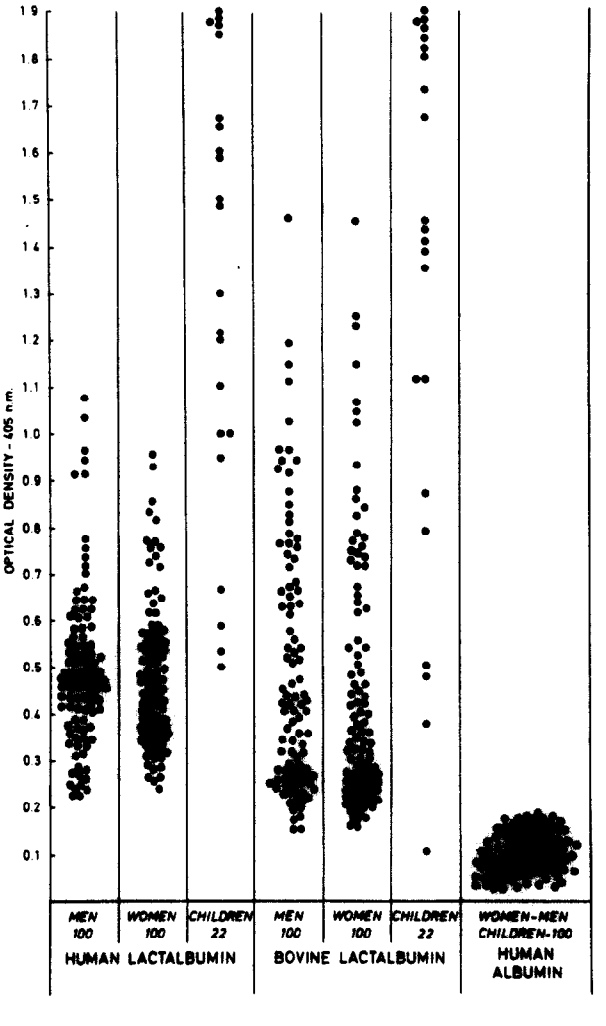

Fig. 3. Enzymoimmunological demonstration of anti-lactalbumin antibodies in the sera of men, women and children. Ordinate: optical density at 450 nm following incubation of sera in wells coated with human albumin (control) and bovine and human lactalbumin.

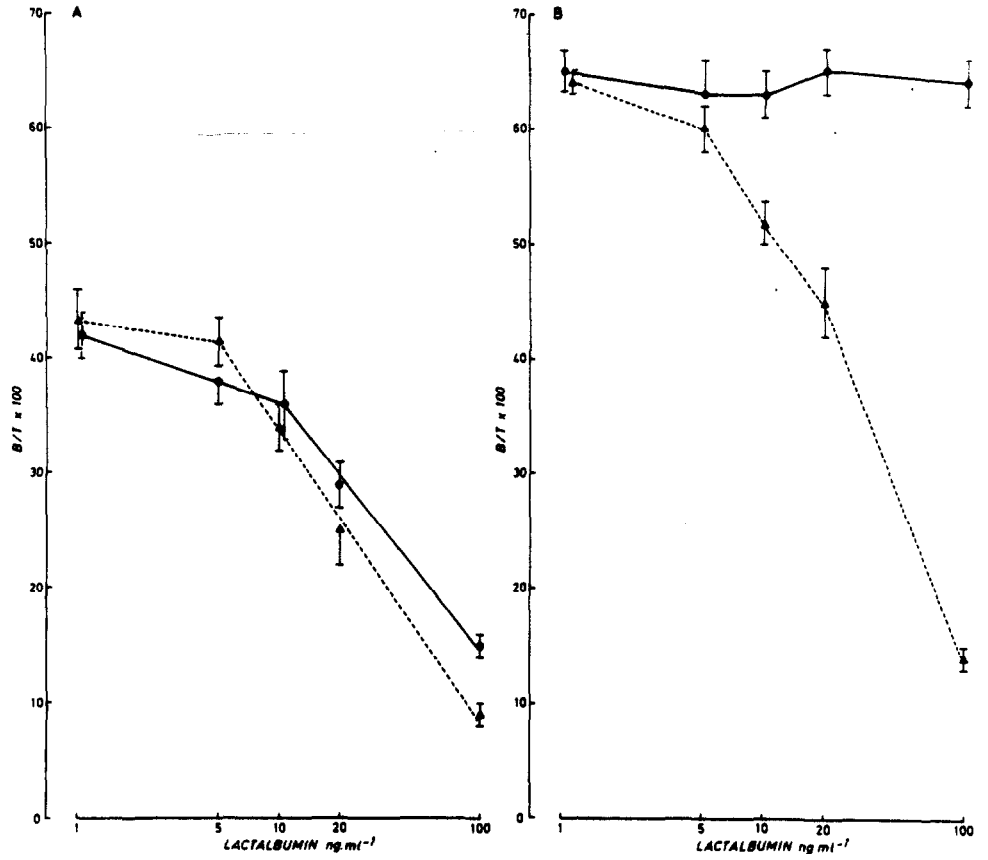

Fig. 4. Displacement of labelled human lactalbumin (panel A) and labelled bovine (panel B) bound to human immunoglobulins G by increasing quantities of human (●—●) and bovine lactalbumin (▲----▲) added to the incubation medium. Ordinate: ratio of radioactivity bound to antibody to total radioactivity.

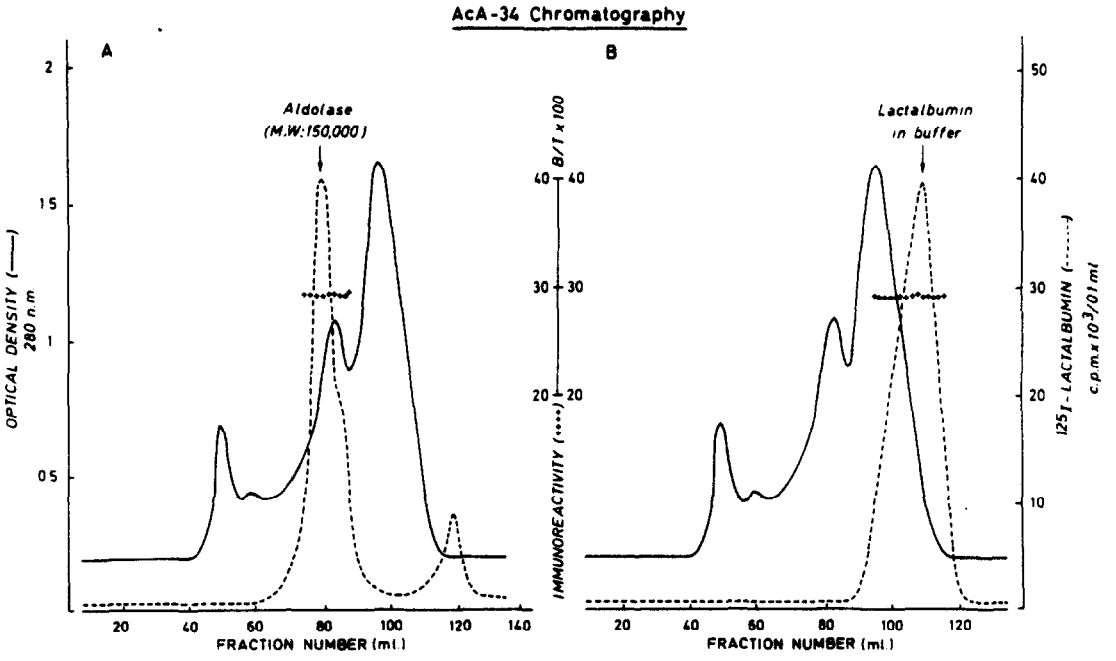

Fig. 5. Chromatography of ultragel Aca 34 (100 × 1.5 cm) of labelled human lactalbumin previously incubated in 1 ml of human serum containing substantial quantities (panel A) or little antilactalbumin immunoglobulin (panel B). Abscissa: elution volume (ml). Ordinate: left: optical density 280 nm (——); right; radioactivity in cpm/0.1 ml (-----); middle: immunoreactivity (B/T) (+++++). Area of elution of molecular weight tracer.

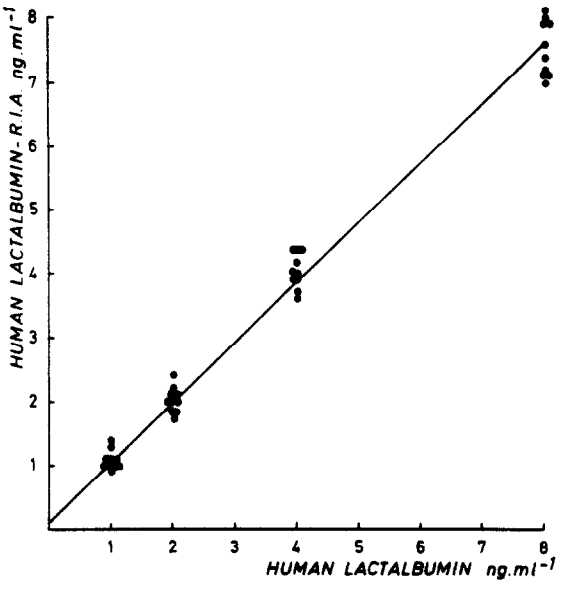

Fig. 6. Linear regression between the amount of lactalbumin incubated (abscissa) and that measured by radioimmunoassay (ordinate).

displacement of less than 25% of labelled human lactalbumin. There thus appear to be two populations of antibodies in our rabbit antiserum: the larger amount which reacts only with human lactalbumin and a smaller population reacting both with human and bovine lactalbumin. A similar weak and incomplete cross-reactivity was found by other authors [15]. On the other hand, bovine lactalbumin did not appear to interfere in the human lactalbumin assay described by Stevens *et al.* [12].

Serial dilutions of sera from pregnant and lactating women completely inhibited the labelled human lactalbumin–antibody reaction with a displacement curve parallel to that obtained with unlabelled lactalbumin. These biological fluids therefore contain an immunoreactive substance ressembling human lactalbumin and detected by the radioimmunoassay.

Using enzymoimmunoassay we have shown that virtually all 222 human sera tested from normal subjects contain a circulating human immunoglobulin of the IgG type which binds bovine and human lactalbumin, and that the sera from children have greater reactivity. It is therefore important before establishing a radioimmunoassay to look to the quality of these antibodies and to their possible interference in the dosage.

By a radioimmunoassay method we have shown that these immunoglobulins G were directed mainly against bovine lactalbumin. For the same tracer mass, the binding of labelled bovine lactalbumin was one and a half times greater than that of labelled human lactalbumin. The maximum affinity constant for bovine lactalbumin was four and a half times greater than that for human lactalbumin. Displacement studies using unlabelled lactalbumin showed that there were two populations of antibodies in human serum: the first reacted only with bovine lactalbumin and showed no displacement with human lactalbumin, while the second reacted with both human and bovine lactalbumin.

The correlation between the amounts added and first incubated in sera containing anti-bovine lactalbumin immunoglobulins G and the amounts measured by radioimmunoassay was close to 1 (0.989). This lack of interference was presumably due to the higher affinity constant of our rabbit antiserum for human lactalbumin — 22.5 times greater than that found for the highest affinity constant of human immunoglobulin G.

This interpretation is supported by the comparable immunoreactivity of tracer eluted in the immunoglobulin region or in the region of lactalbumin after it had been incubated with sera containing large or small quantities of endogenous immunoglobulin G and subsequently chromatographed on Aca 34.

The presence of these immunoglobins G did not interfere in our radioimmunoassay, which can therefore be used for looking for the significance of this milk protein in the serum. The lack of this important control by different authors [3, 8, 10, 11, 14] explains the wide difference in the results obtained and could re-establish the value of this protein as a functional marker for the normal and pathological activity of the breast [7].

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