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α-Lactalbumin Production in Human Mammary Carcinoma

Abstract. *α-Lactalbumin was isolated from mature human milk and utilized as an immunogen in rabbits. A radioimmunoassay was developed that was capable of detecting nanogram quantities of the antigen. α-Lactalbumin synthesis was detected in human breast cancer cells (MCF-7) cultivated as a continuous cell line in vitro. Other human carcinoma epithelial cell lines (throat and cervix) failed to react in this assay. The ability to synthesize α-lactalbumin by breast carcinoma cells appeared to be independent of the addition of prolactin to the culture medium.*

α-Lactalbumin (α-LA) is a protein specifically synthesized in functionally differentiated mammary epithelial cells. It is recoverable in large quantities from their secretory products, milk and colostrum. The protein has been identified by Brodbeck and Ebner (*1*) as the B protein of lactose synthetase (E.C. 2.4.1.22) which catalyzes the formation of lactose.

$$\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP} \quad (1)$$

In the absence of α-LA, the A protein of lactose synthetase is a galactosyltransferase utilizing *N*-acetylglucosamine or a terminal *N*-acetyl unit on an oligosaccharide moiety of a glycoprotein (*2*)

$$\text{UDP-galactose} + N\text{-acetylglucosamine} \rightarrow N\text{-acetyllactosamine} + \text{UDP} \quad (2)$$

This galactosyltransferase is found predominantly in the mammary gland, but has been isolated from most tissues (*3*). α-Lactalbumin functions as a "specifier" protein, in that it alters the substrate specificity of the A protein, thus regulating an organ-specific reaction, lactose synthesis (*4*).

In our study, α-LA was isolated from whole, human milk by a modification of the procedures published by Nagasawa *et al.* (*5*). Skim milk at 25°C was adjusted to *p*H 4.6 with 0.1*N* HCl, and the precipitated casein was removed by centrifugation at 15,000*g* for 20 minutes. The *p*H of the clear supernatant (whey) was raised to 7.0 with 0.1*N* NaOH, and the solution was concentrated tenfold in an Amicon ultrafiltration cell with a UM-2 membrane. Percolation through a Sephadex G-100 column equilibrated with 0.01*M* imidazole-hydrochloride buffer (*p*H 7.0) resulted in the separation of α-LA from most of the whey proteins (Fig. 1A). After concentration in the Amicon ultrafiltration cell, homogeneous α-LA was eluted from a DEAE-cellulose column with a linear NaCl gradient in 0.01*M* imidazole-hydrochloride buffer, *p*H 7.0 (Fig. 1B). The eluate was concentrated, filtered under pressure against distilled water until free of chlorides, lyophilized, and stored at 20°C.

The isolated α-LA was judged to be homogeneous on the basis of alkaline (*p*H 8.9) disc electrophoresis, 0.2 percent sodium dodecyl sulfate gel electrophoresis (*p*H 7.8), and acid (*p*H 4.3) disc electrophoresis over a 35-fold protein concentration range (7 to 240 μg). In each instance only a single protein band was observed after staining with Coomassie brilliant blue. Acrylamide gel isoelectric focusing (*6*) (*p*H 3 to 10) also yielded a single band. The α-LA failed to react in Ouchterlony double diffusion against goat antiserum to human serum and rabbit antiserum to human casein.

Amino acid analyses were performed on a 6*N* HCl hydrolyzate (Technicon amino acid analyzer) (*7*). The amino acid analyses were in excellent agreement with the recorded values for α-LA, based on the amino acid sequence, indicating freedom from casein contamination (*8*).

Antibodies against human α-LA were produced in male New Zealand white rabbits by immunization with purified α-LA. The α-LA (5 mg) was dissolved in 0.5 ml of

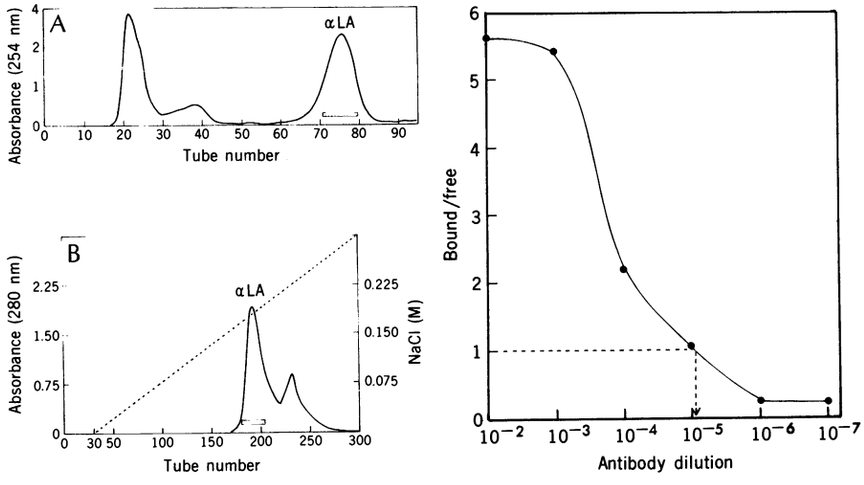

Fig. 1 (left). (A) Separation of α-LA from human whey proteins. Whey proteins (150 mg) were separated on a column (5 by 100 cm) of Sephadex G-100 (fine) equilibrated with 0.01*M* imidazole-hydrochloride buffer, *p*H 7.0. The flow rate was 60 ml/hour; 15-ml fractions were collected. The fractions indicated with the horizontal bar were pooled and concentrated by ultrafiltration. (B) Diethylaminoethyl-cellulose chromatography of α-LA. DEAE-cellulose (0.9 meq/g, lot 11140, Bio-Rad Laboratories) was equilibrated with 0.01*M* imidazole-hydrochloride buffer (*p*H 7.0) and packed into a column (2.5 by 85 cm); 150 mg of α-LA isolated from (A) was placed on the DEAE-cellulose. The column was then washed with starting buffer. The α-LA was eluted with a linear gradient formed from 1000 ml of 0.01*M* imidazole-hydrochloride (*p*H 7.0) and 1000 ml of the same buffer containing 0.30*M* NaCl (*p*H 7.0). The flow rate was 40 ml/hour, and 10-ml fractions were collected. Fig. 2 (right). Titration curve of rabbit antiserum to human α-LA with ^{125}I-labeled α-LA. The diluent for all radioimmunoassay reagents was PBS containing EDTA (1 m*M*), disodium salt, and bovine serum albumin (1 mg/ml). The sodium phosphate buffer was 0.05*M* and NaCl was 0.10*M* with a final *p*H of 7.4. A series of tenfold dilutions ranging from 10^{-2} to 10^{-7} of rabbit antibody to human α-LA was made in diluent buffer. Each serum dilution (50 μl) was reacted with 0.5 ng (10 μl) of ^{125}I-labeled α-LA antigen in the presence of 128 μg of normal rabbit gamma globulins (final volume, 150 μl); incubation proceeded at 37°C for 2 hours in a Dubnoff metabolic shaking incubator and was terminated by the addition of goat antiserum to rabbit gamma globulins; the tubes were then incubated for another hour at 37°C, and 16 hours at 4°C. The immunoprecipitate was centrifuged at 900*g* for 60 minutes, and the radioactivity of a 100-μl sample of supernatant (unbound ^{125}I-labeled α-LA) counted in a liquid scintillation spectrometer, calibrated for ^{125}I (*6*).

Table 1. Effect of prolactin on α-lactalbumin synthesis.

Human cell lines	Culture		Protein (μg/assay)	α-LA	
	Hormones	Prolactin exposure (days)		ng/mg protein	10^5 molecules/cell
MCF-7	I*	0	34.5	207	1.62
	I	8	39.0	140	1.09
	IFP	8	35.5	195	1.40
	I	12	52.5	190	1.36
	IFP	12	60.0	140	1.09
	I	16	34.0	166	1.19
	IFP	16	44.5	140	1.09
HeLa	I	10	140	0†	
	IFP	10	142	0	
D-562	I	10	146	0	
	IFP	10	146	0	

*Insulin (I), cortisol (F), prolactin (P); used at a concentration of 10 μg/ml. †Minimum detectable level, 0.150 ng of α-LA per assay.

phosphate buffered saline (PBS), *p*H 7.4, and emulsified with an equal volume of Freund's complete adjuvant. This dose was injected into each of three subdermal sites (at the nape of the neck and behind each hind leg) weekly for 4 weeks. Booster injections containing 2.5 mg of protein in 1.0 ml of isotonic saline were administered 10 days before the animals were bled. A bleeding at 6 months yielded the serum used for further experimentation.

The antiserum was monospecific by Ouchterlony double diffusion and immunoelectrophoresis when tested against purified α-LA and concentrated whey. There was no evidence of reactivity against human serum proteins or human casein.

Carrier-free ^{125}I was coupled to α-LA by the chloramine-T method (*9*). Unbound isotope and reaction products were separated from the ^{125}I-labeled protein by passage through a Sephadex G-25 (fine) column (1.5 by 28 cm) equilibrated with PBS, *p*H 7.4. The iodinated protein was located in the column eluent by monitoring radioactivity and ultraviolet absorbance at 225 nm. The material with the highest specific activity was submitted to pressure filtration in an Amicon cell with a UM-2 membrane in order to remove additional unbound radioactive iodine. The specific activity of the iodinated protein ranged from 130 to 250 c/mmole. Specific activities greater than 350 c/mmole resulted in total loss of immunoreactivity.

Rabbit antiserum to human α-LA was titrated with ^{125}I-labeled antigen in order to determine the dilution of antiserum required to give 50 percent binding (Fig. 2). Forty-five to 50 percent binding was achieved at a final antibody dilution of 1 : 109,000. The quantity of human α-LA in an unknown sample was determined by competitive inhibition assays, where unlabeled antigen in the test sample competed against labeled antigen for binding to the antibody, thereby diminishing the binding of the labeled antigen.

The specificity of the antibody was examined by competitive binding studies with (i) lactalbumins isolated from the milk of six animal species (*10*) and (ii) various proteins of a similar size and hydrodynamic shape. None of the lactalbumins cross-reacted significantly, on the basis of the quantity of animal lactalbumin required to inhibit 50 percent of the binding (I_{50}). Human α-LA required 0.24 ng as compared with bovine (5.2 μg), rat (9.0 μg), dog (20 μg), and goat α-LA's (45 μg). Gerbil (25 μg) and pig (50 μg) lactalbumins only achieved a maximum of 23 percent displacement. An important aspect of this assay is that lysozyme, a normal constituent of human epithelial cells and a protein that shows a large degree of amino acid homology with human α-LA (*11*), does not interfere with the assay at all, even when added in 100,000 molar excess. Similar results have been obtained with human serum proteins, egg white lysozyme, and bovine pancreatic ribonuclease A. The data from the last two have been omitted from Fig. 3.

A human cell line (MCF-7) cultivated at the Michigan Cancer Foundation was derived from a pleural effusion of a patient with metastatic breast adenocarcinoma (*12*). Results of our studies suggested that MCF-7 were human cells (*12*). Brooks *et al.* demonstrated that MCF-7 cells exhibited specific nuclear and cytoplasmic 17β-estradiol receptors (*13*). To determine whether MCF-7 cells synthesized α-LA and, therefore, retained some features of differentiated mammary epithelium, these cells as well as two human epithelial nonbreast-derived control cells were subjected to radioimmunoassay.

All three human carcinoma cell lines, MCF-7, D-562 (throat) (*14*), and HeLa (cervical), were cultured in Falcon T-75 flasks with Eagle's minimal essential medium in Hanks salt solution supplemented with 5 percent calf serum and insulin (10 μg/ml). In several experiments cortisol and prolactin (ovine) were also added to the culture media. Particulate-free supernatants were prepared from homogenized harvested cells by centrifugation at 600*g* to

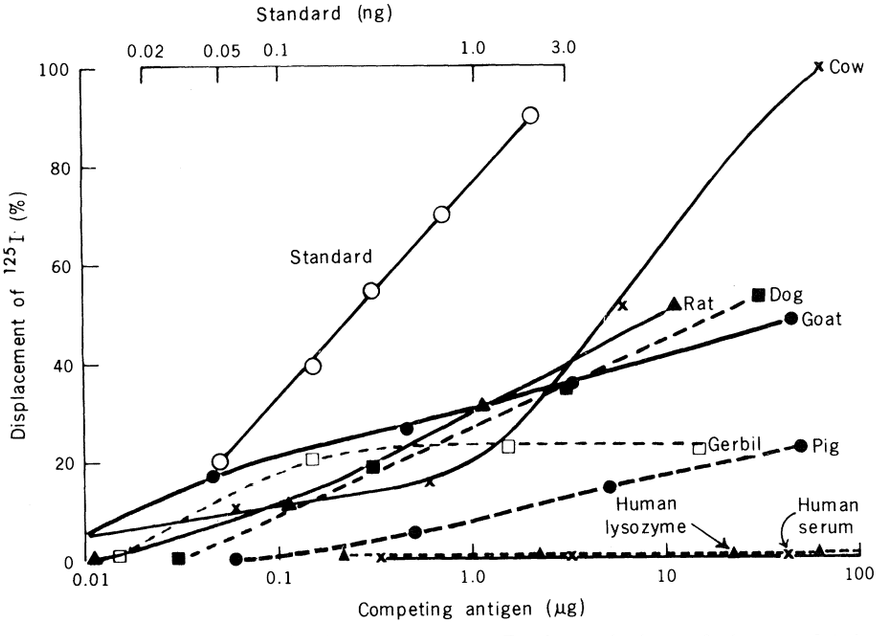

Fig. 3. Radioimmunoassay binding displacement curves. For the standard curve for human α-LA the reagents were placed in glass tubes (12 by 75 mm) in the following order: 10 μl of ^{125}I-labeled α-LA (0.5 ng), 40 μl of normal rabbit gamma globulins (128 μg), 50 μl of unlabeled α-LA standard solutions (0.020 to 20 ng). The contents of the tube were mixed, and 50 μl of rabbit antiserum to α-LA was diluted to a concentration that would bind 50 percent of the available labeled antigen (final volume, 150 μl). Incubation, precipitation, and counting were as described in the legend to Fig. 2. Serum was from an adult nonpregnant premenopausal woman.

remove cellular debris, followed by ultracentrifugation at 105,000*g*.

Analysis of these supernatants by radioimmunoassay indicated that neither the malignant throat cells nor the malignant cervical epithelial cells in any hormonal milieu used were capable of detectable α-LA synthesis. MCF-7 cells, however, synthesized readily measurable quantities of α-LA. The amount of α-LA synthesized by MCF-7 cells in insulin-containing media corresponded to 200 ng per milligram of soluble cell protein in 20×10^6 cells. This quantity did not increase significantly when either cortisol or prolactin was added to the medium for periods up to 16 days (Table 1). The results of other experiments in which α-LA synthesis was measured both as a function of prolactin concentration and duration of prolactin exposure also indicated no stimulation of α-LA synthesis in the presence of prolactin. The fact that calf serum was used to supplement the growth media, which could contain sufficient endogenous prolactin to stimulate α-LA synthesis, prevents rigorous exclusion of a prolactin effect. Experimentation in which prolactin was added to serum-free chemically defined medium (*15*) has not resulted in increased α-LA synthesis.

The immunochemical data in our study provided further evidence that the MCF-7 cell line is mammary and epithelial and that the capacity to synthesize α-LA was not lost as a function of malignant transformation. An alternative to that interpretation is that MCF-7 cells are not breast epithelial, but only acquired α-LA synthesizing ability ectopically in culture. However, MCF-7 cells exhibit other markers of breast epithelium (*12, 13*) and the probability of coordinate synthesis of breast receptor proteins and α-LA in nonbreast cells is considered remote. Should α-LA synthesis be present in a significant number of other cultured breast carcinomas, the assay would then serve as an additional marker to identify neoplastic cell lines of questionable phenotype as breast epithelium.

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Sensitive Period for the Development of Human Binocular Vision

Abstract. *Twenty-four subjects with abnormal binocular experience, due to a condition of convergent strabismus that existed during different periods of their lives, were tested. Interocular transfer of the tilt-aftereffect was used to assess binocularity. Individuals between 1 and 3 years of age are most susceptible to abnormal binocular experience.*

The structure and function of the mammalian visual system are greatly affected by abnormal visual experience, provided that the abnormal experience occurs during a specific period of development. Several properties of visual cortical neurons are affected by abnormal experience during this critical or sensitive period (*1, 2*). Kittens deprived of visual input in one eye fail to develop a significant number of binocular cortical neurons if the monocular deprivation occurs during the period beginning at age 4 weeks and ending at age 12 weeks (*2*). Experimentally induced strabismus (misalignment of the visual axes) also results in a failure to develop cortical binocularity if the strabismus occurs during that 4- to 12-week period (*3*). Thus, adult cortical binocularity in the cat is dependent on concordant binocular visual experience during the sensitive period.

The development of the human visual system is also affected by early visual experience (*4*), but the period of greatest susceptibility to abnormal experience has not been determined. We have demonstrated the existence and estimated the time parameters of a sensitive period for the development of binocularity.

We tested 24 human subjects who have

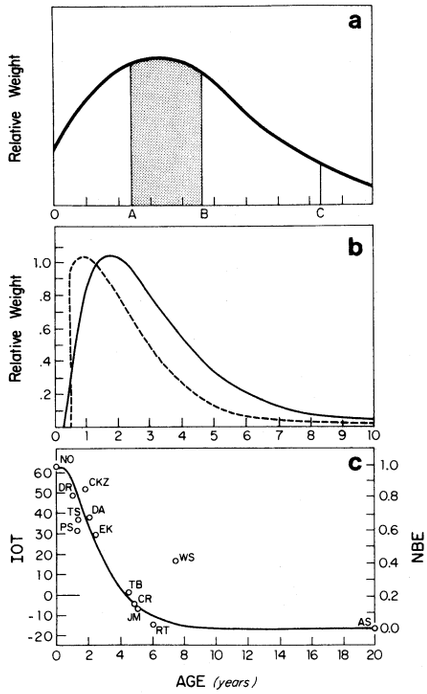

Fig. 1. (a) Method for describing the sensitive period for the development of binocularity. The curve represents one form of the arbitrary four-parameter function (*10*). For subjects with a history of esotropia, the area under the curve from the age of onset of esotropia (*A*) to the age at surgical correction (*B*) was subtracted from the area under the curve from birth 0 to the age at which we tested them (*C*). The resultant value for each subject is an estimate of the amount of normal binocular experience (NBE) which that subject encountered. (b) Developmental weighting functions which yielded the highest correlations between interocular transfer (IOT) and NBE. The solid line represents the best-fitting function for 12 congenital esotropes, and the broken line, that for 12 late-onset esotropes. These functions indicate the relative importance of abnormal binocular experience from birth to age 10. (c) Point plot of the IOT values as a function of the age at corrective surgery, for each of the 12 congenital esotropes. The solid line represents NBE for congenital esotropia [derived from the function in (b)] as a function of age at surgery (*13*).