STUDIES ON THE RELATIONSHIP OF α-LACTALBUMIN AND MAMMARY CARCINOMAS

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STUDIES ON THE RELATIONSHIP OF α-LACTALBUMIN

AND MAMMARY CARCINOMAS

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ABBREVIATIONS

α-LA - α-Lactalbumin

RIA - Radioimmunoassay

All other abbreviations are according to the Journal of Biochemistry format.

CHAPTER I

INTRODUCTION

Mammary cancer currently kills 30,000 people each year in the United States and the number is growing (1). As with most cancers, probably no one cause is responsible for the many different types of malignant and benign breast diseases. Since attempts to link vertical transmission of a virus with mammary cancer have as yet failed, the hope for a quick cure by a polio-type immunization with an attenuated virus has diminished. The best chance for survival still rests with early detection, mastectomy, irradiation, chemotherapy, and immunotherapy.

Prevention and more effective treatment are clearly dependent upon a better understanding of the biochemical errors that produce the state termed cancer. This requires careful analysis of human breast diseases to determine whether such factors as estrogen and prolactin receptors correlate with hormonal sensitive growth, whether embryonic proteins become re-expressed, and most importantly whether or not the differentiation of breast diseases encompasses a wide spectrum. If a wide range of biochemical differentiation is found, the general methods currently used to treat pre- and post-menopausal breast lesions may need to be modified to suit the individual tumor.

The development of animal models that closely resemble the human situation is, for obvious reasons, extremely important. In

order to assess the biological status of mammary epithelial cells in both animal and human tumors, it is necessary to utilize indicators which are unique for differentiated mammary epithelial cells. The indicator must be found in measurable amounts, must be stable, and purifiable to homogeneity. These requirements are fulfilled by α-lactalbumin. This protein, totally unique to mammary epithelial cells, is found in high concentration in the secretory cells only during lactation, and follows the cyclic production of milk from preparturition through regression (2). In 1964 Ebner and Brodbeck (3) first described the biological function of α-LA, which serves as a modifier protein for a galactosyltransferase by lowering the K_m of glucose from molar to millimolar so that lactose production can proceed at a significant rate. Enzymatic measurement of low levels of α-LA by incorporation of radioactive galactose from UDP-galactose into lactose proved unsatisfactory in tissues due to the presence of enzymes which hydrolyze UDP-galactose. To circumvent these problems, radioimmunoassays for human and rat α-LA were developed. These assays provide a thousand fold increase in sensitivity, are specific for α-LA, are very reproducible, and are accurate. By measuring the level of α-LA in breast lesions, one can estimate the degree of lactation, or in other terms, estimate the state of differentiation of the tissue. Metastases to different tissues can be compared with the primary tumor and thereby provide information on the state of differentiation required by the metastasizing process. Other breast diseases such as benign fibrocystic disease, gynecomastia, and galactorrhea can be examined in order to determine if these diseases should be considered as valid precancerous states.

Since α-LA appears in the blood during lactation, it is of interest to determine the feasibility of using elevated serum or urinary α-LA levels to screen populations for early detection of breast lesions which produce α-LA. In addition, it might be possible to evaluate the effectiveness of surgery which attempted to remove all the primary focus and metastases.

There have been many attempts to develop model systems for human breast cancer. The Mason Tumor Bank maintains a number of the successfully transplanted animal tumor lines, but the only distinction made at present is based upon histological appearance. Numerous attempts have been made to establish and maintain human mammary carcinoma cells in cell cultures, and a number of such cultures are available. Recently, there have been claims based upon karyotypical and isoenzyme markers that these lines have been contaminated and eventually overgrown by Hela cells (4). Measurement of α-LA levels could help to establish the parentage and degree of differentiation of these and other cell lines.

Immunofluorescence and the horseradish peroxidase techniques provide other methods of localizing and detecting α-LA in tissue sections. Much controversy exists in several breast diseases, such as Paget's disease and ductal/lobular carcinomas with respect to the parentage and differentiation of the malignant cells. Since isolation of these cell types is impossible, localization of α-LA within a cell population of a tissue would be clinically very beneficial.

Every mammal's milk contains immunoglobulins, yet mammary epithelial cells do not synthesize γ-globulins. Instead, γ-globulins are produced in the blood by the plasma cells and by some mechanism are transported through the mammary epithelial cells and secreted with

the milk. The process is selective for certain γ-globulins and is a form of active transport since milk γ-globulin concentrations can be higher than serum γ-globulin levels. By directly labeling pure γ-globulins with fluorescein, one can investigate the extent of γ-globulin receptors on cells and their specificity.

In this study, radioimmunoassays were developed for rat and human α-LA and levels of α-LA were determined in rat and human tumors in order to assess the value of this assay for tumor detection. In addition, immunofluorescence and peroxidase coupled antibodies to α-LA were used to localize α-LA in tissue sections. Such assays were complicated by the presence of γ-globulin receptors on the mammary epithelial cells and this was circumvented by pretreatment with formalin or blocking γ-globulins.

CHAPTER II

LITERATURE REVIEW

Hormonal Regulation of Gene Expression in Mammary Cells

In order to designate the biochemical lesions in breast cancer, it is necessary to understand the intricate and unique biochemistry of the normal alveolar cell. During pregnancy the mammary gland undergoes remarkable developmental changes related to the formation of an exocrine gland that will form and secrete the specialized components of milk after parturition. This complex process of growth and differentiation is regulated by a large number of intracellular molecular mechanisms, which in turn are governed by a complex set of specific hormonal signals.

Growth of the mammary gland proceeds through a process of cell proliferation rather than by hypertrophy of existing cellular units (5). Several hormones have been found to regulate the proliferation of mouse midpregnant mammary epithelial cells in organ culture. Time course studies of DNA synthesis in midpregnant mammary explants showed that insulin stimulates an approximate three-fold increase in the rate of synthesis, with the maximal rate observed at about 24 hours; and after a partially synchronized wave of DNA synthesis, cellular proliferation virtually ceases after about 96 hours of incubation. This stimulation represents an increase in the number of epithelial

cells induced to replicate DNA as indicated by mitotic indexes. Preparations of pituitary growth hormone and epithelial-epidermal growth factor also induce an increase in the number of epithelial cells to enter the S (DNA synthesis) phase of the cell cycle (6). In contrast to the single wave of DNA synthesis induced by insulin, epidermal growth factor, or growth hormone, the addition of serum to the chemically defined medium induces several waves of DNA synthesis which continue for prolonged periods of culture (7). The activity in serum which induces the proliferation has been partially purified from human serum, and appears to be a heat labile, low molecular weight polypeptide whose activity is not neutralized by antisera which inactivate insulin, growth hormone, or epidermal growth factor. The partially purified factor is effective at even 10% of its normal serum concentration. In contrast, the minimal effective concentration of insulin in vitro is about 50 fold greater than normal physiological conditions. Although epidermal growth factor is effective at normal serum levels, it has been detected only in the serum of the mouse, rat, and rabbit (8). Because of this narrow species distribution, epidermal growth factor may not be of general importance in the regulation of mammary gland growth.

Studies on the effect of estradiol-17β on cell proliferation in mid-pregnant explants have indicated a possible role for this hormone in the regulation of mammary epithelial cell proliferation. Addition of estradiol-17β to the serum-free chemically defined culture media has little effect on cell proliferation in the absence of insulin. However, under conditions in which cells are induced to initiate DNA synthesis, such as in the presence of insulin, estradiol-17β is found

to exert a concentration dependent permissive effect (9). At 10^{-12} M concentration, estradiol-17β markedly inhibits DNA synthesis, mitotic activity, and the induction of DNA polymerase. As the concentration of estradiol-17β is increased over the physiological range (10) to a concentration of 10^{-10} M, DNA synthesis increases to approximately the maximal rate induced by insulin. Autoradiographic studies demonstrated that these effects relate to the numbers of cells which enter the S phase rather than to the rate of DNA replication per cell. The inhibitory concentration of estradiol-17β is similar to serum levels before puberty, a period in which mammary gland growth is largely prevented. The higher 10^{-10} M concentration is within levels found in the sera of adult women (11) and appears to permit maximum rates of induced cell proliferation. Mammary growth at puberty may, therefore, be mediated by a permissive effect of increasing serum concentrations of estradiol-17β by modifying the rate at which cells pass from the G phase into the S phase, although it does not seem to be a sufficient hormonal stimulus in itself to induce the initiation of DNA synthesis.

In addition to the initiation of DNA replication, a number of other biosynthetic events related to chromosomal replication are induced in mammary epithelial cells in organ cultures containing insulin. After about 16 hours of incubation the maximum rate of incorporation of ^{3}H amino acids into acidic chromatin is observed, and about eight hours later maximal rates of DNA synthesis, methylation of nascent DNA chains, DNA polymerase activity, and ^{14}C amino acid incorporation into histones are seen (12).

Hormonal induction of milk proteins has been studied in depth by using explants from midpregnant mouse mammary glands which contain

two types of epithelial cells: secretory alveolar cells and undifferentiated stem cells. Autoradiographic techniques have demonstrated that the secretory alveolar cells in the gland of pregnant or lactating mice seldom divide (13). In contrast, the undifferentiated epithelial cells can be induced to divide by the previously stated methods. Tissues incubated in medium containing insulin, hydrocortisone, and prolactin are found to contain increased numbers of secretory alveolar cells, and to exhibit increased synthesis of milk proteins---a characteristic and unique activity of mammary alveolar cells (14). A characteristic sequence of hormonal actions is required for the induction of the milk proteins casein and lactose synthetase (15, 16, 17, 18, 19, 20). Several hormones may serve to regulate the rate of cell division among the undifferentiated stem cell population. A period of stimulation with hydrocortisone is required before milk protein synthesis can occur in the new daughter cells. In cells thus pretreated with insulin and hydrocortisone, prolactin or placental lactogen can synergize with insulin to induce milk protein formation (21, 22, 23). A characteristic sequence of molecular biosynthetic events occurs upon addition of prolactin to the medium. All of the increases in rates of synthesis depend upon prior completion of cell division, previous treatment with hydrocortisone and the concomitant, synergistic action of insulin. During the first 20 minutes following the addition of prolactin, a net increase in the rate of synthesis of rapidly labeled nuclear RNA and preribosomal RNA can be observed (24), followed by increased incorporation of radioactivity into ribosomal RNA and finally increased numbers of polysomes in the cytoplasm of the mammary epithelial cells (25). After four to six hours of incubation with prolactin, the

synthesis of all the components of casein is stimulated coordinately. After 12 hours of incubation, increases in the enzymatic activities of the two components of lactose synthetase, galactosyltransferase and α-lactalbumin, are observed. (4). The observation that the prolactin-induced stimulation of milk protein synthesis is completely prevented by inhibitors of RNA synthesis, such as actinomycin D and mitomycin C, represents evidence that is consistent with the concept that the induction of these specific proteins is dependent upon the preceding increases in RNA formation.

Differentiation of mammary epithelial cells during pregnancy in the mouse is associated with a marked increase in transfer RNA (tRNA) as indicated by amino acid acceptor activity and tRNA methylases (26). Formation of active tRNA is completed by methylation of the polynucleotide RNA chain by a group of specific methylating enzymes. Characterization of hybridizable RNA in lactational cells has demonstrated that these cells contain species of RNA which are not detectable in the undifferentiated cells by RNA-DNA hybridization competition experiments (27, 28).

Demonstration that the hormone dependent differentiation of mammary epithelial cells is associated with marked activation of gene transcription which results in the formation of nRNAs that are not detectable in undifferentiated cells is consistent with the concept that hormone-dependent cell differentiation represents the derepression of specific genes in the differentiated cells which were not transcribed in the undifferentiated stem cells.

To evaluate the potential role of specific proteins in the regulation of gene activation and transcription, the synthesis of specific

nuclear proteins was studied by Marzluff et al. (29). Studies on mammary epithelial cell histones have demonstrated that these proteins lack tissue or species specificity and therefore seem unlikely to contain the structural specificity anticipated for the recognition and regulation of specific nucleotide sequences of genes. In contrast, studies on mammary acidic chromatin protein have demonstrated that these proteins are extremely heterogeneous and are tissue and species-specific, and could represent a class of proteins involved in the activation of specific genes. In other tissues this class of proteins has been reported to bind to the DNA of the tissue of origin only, and to modify the sequences of hybridizable RNA which are transcribed on reconstituted chromatin (30). Acrylamide gel electrophoresis in sodium dodecyl sulfate of highly purified preparations of acidic chromatin protein from virginal glands, and lactating mammary glands and liver reveal a complex of six proteins with low electrophoretic mobilities in the lactational tissue which are not detectable in the preparations from undifferentiated mammary tissues or other mouse tissues (31). Incubation of developing mammary gland explants with insulin or serum causes a marked stimulation of incorporation of radioactivity into nearly all the acidic chromatin proteins, but incubation with hydrocortisone and/or prolactin does not significantly affect the net rate of synthesis or turnover of these specific proteins. Thus, the synthesis of these acidic chromatin proteins is markedly altered by factors that affect cell proliferation, but is not regulated by the hormones which induce milk protein synthesis in differentiated alveolar cells (32). These observations suggest a tentative model for initial or early gene activation in mammary cell differentiation.

Induction of DNA replication in the undifferentiated stem cells by either insulin or serum factor, with the possible permissive effect of estradiol-17β, is associated with the formation of new and unique acidic chromatin proteins which are specific for differentiated and lactation-inducible cells. The replication of DNA may thus represent the critical time during which new histones and acidic proteins may recombine with DNA in such a manner as to permit a new pattern of gene derepression in the daughter cells into which is segregated the newly snythesized DNA with its new unique nuclear proteins. Insulin or the serum factor thus appear to represent hormonal triggers which set in motion a sequence of intrinsic mechanisms which may lead to altered patterns of gene expression.

Following this insulin-induced division-differentiation, a large number of molecular responses are observed in mammary epithelial cells after stimulation with prolactin. The first action of prolactin on its target cell appears to be the binding of prolactin to a hormone specific receptor site. Utilizing a binding assay with mouse mammary epithelial cell particles, Turkington (33) reported a rather prolactin-specific saturable binding activity with a dissociation constant of 9×10^{-9} M which had only about 27% displacement with bovine growth hormone. Specific binding activities in particles prepared from various organs of the mouse and rat showed the highest specific binding in lactating mammary gland with about one-third that binding in mid-pregnant mammary glands, liver and kidney, and about one-tenth that binding in adrenal, ovary and seminal vesicles. No saturable binding activity was found in tissues where no physiological activity of prolactin is known such as heart, lung, spleen, and cerebral cortex (34).

These data integrate well with prolactin's known ability to increase sodium readsorption in kidneys and its regulation of hepatic lipogenesis in migratory birds, as well as a trophic influence on the adrenal gland, and may suggest additional organs in which it may play a role (35). The prolactin receptor in mammary particles is degenerate in that it recognizes all lactogenic hormones, as seen by the ability of growth hormone and placental lactogen to displace prolactin from receptors, although prolactin is the most potent of the lactogenic hormones. The chemical nature of the prolactin binding activity in mouse and rabbit mammary gland particles was investigated by pretreatment with various enzymes, and binding was significantly decreased by treatment with trypsin and phospholipase-C, but was unaffected by collagenase, neuraminidase, RNase and DNase (36). A recent report by Chomczynski and Topper (37) indicated that placental lactogen and prolactin can stimulate the rate of RNA synthesis by nuclei isolated from mammary epithelial cells and suggested that these protein hormones may perform some of their functions within the mammary cell.

Early studies on the hormonal regulation of mouse mammary epithelial cells demonstrated that ^{32}phosphorous was incorporated into specific histones and acidic nuclear proteins which were temporally associated with activation of transcription by insulin and prolactin (25). Because the phosphorylation represented early and dramatic effects of prolactin on DNA-associated proteins, investigators were interested in determining the mechanism by which protein phosphorylation is regulated in mammary epithelial cells. Protein kinase activity which exhibited a high substrate specificity for serine and threonine residues of nuclear proteins was purified from lactating mammary gland

by Majumder and Turkington (38). Two forms of the enzyme, both found in the cytosol, were resolved, with protein kinase II exhibiting the important property of activation by cyclic nucleotides. The apparent K_m value for cAMP was 0.03 μM, whereas activation with other cyclic nucleotides was in the range of one to three μM. Protein kinase II was found in association with a specific cAMP-binding protein which could be dissociated by incubation with cAMP into a cAMP-binding subunit and the activated catalytic subunit which is identical to protein kinase I. Levels of both protein kinases in mammary epithelial cells during cellular differentiation in vivo change coordinately, increasing dramatically during the first half of pregnancy until at parturition they exhibit a nine fold maximum over virginal tissue. In contrast to the hormonal induction of the milk proteins, which requires the action of insulin, hydrocortisone, and prolactin, the activities of the catalytic and regulatory subunits of protein kinase were increased by prolactin in cells previously treated only with insulin. Furthermore, the stimulation of the cAMP-binding activity by prolactin did not require the synergistic action of insulin, and in all cases, could be prevented by addition of actinonycin D or cyclohexamide to the culture at the same time prolactin was added. This suggests that induction of the kinase enzymes requires concomitant synthesis of RNA and protein (39).

In order to elucidate the functional role of the hormonally induced protein kinase, the physiological substrates of the enzymes have been studied (40). The rates of phosphorylation of highly purified preparations of mammary cell membranes, ribosomes and histones by exogenous purified protein kinase were compared and histones were

labeled twelve times faster and ribosomal proteins were labeled two times faster than membrane proteins. The endogenous protein kinase activity of purified mammary plasma membranes and ribosomes was heat labile, not extracted by various detergents, and was not stimulated by cyclic nucleotides. The organelle associated proteins that were phosphorylated in mammary explants incubated with insulin, hydrocortisone, and prolactin, were further characterized by polyacrylamide gel electrophoresis and all nineteen phosphoproteins of the membrane showed a marked increase in rate of phosphorylation when compared with membranes from explants grown in only insulin and hydrocortisone. Four of the eight phosphorylated ribosomal proteins showed increased levels of phosphorylation, and these four specific proteins have been observed to increase in the mouse mammary gland during the stages of pregnancy.

Studies were also carried out to correlate the time course of induction of the protein kinase with prolactin-mediated stimulation of protein phosphorylation in vitro. In mammary explants pretreated with insulin and hydrocortisone and subsequently with prolactin, the cAMP-dependent protein kinase II and the cAMP binding activity of the regulatory subunit were rapidly induced to maximal levels in one hour. Subsequently, there were marked increases in the rates of phosphorylation of proteins with the plasma membrane proteins reaching a maximal value at sixteen hours, and nuclear proteins at twenty-four hours. Similar observations were seen for insulin-induced protein kinase (41).

Other highly significant observations concerning the action of prolactin on mammary epithelial cells must be considered before a final model can be assembled. The binding of prolactin to mammary particles does not activate adenylate cyclase in vitro (38). This result suggests

that the prolactin receptor sites may not be functionally organized in the membrane in such a manner as to affect the activity of the membrane-bound adenylate cyclase. Furthermore, since the uptake of large amounts of cAMP from the medium does not serve to substitute for the action of prolactin, it appears unlikely that the generation of cAMP by adenylate cyclase represents a direct step in the action of prolactin (38). A more reasonable model invokes the formation of the prolactin-receptor complex which serves to induce transcription by an unknown mechanism of RNAs necessary to synthesize more protein kinase molecules, which may then serve to propagate the initial action of prolactin throughout the many functionally distinct compartments of the mammary cell by phosphorylating specific protein components of the plasma membrane, ribosomes, and chromatin.

Models for Malignancy

During the last ten years observations have been made that have dramatically altered the general models for malignancy. Broadly organized into two main concepts of environmentally-induced or virally-induced DNA alterations, each theory could account for some aspects of the various types of neoplasms but there were no unifying concepts which would unite the seemingly unrelatable observations. One such perplexing problem was to explain how a virally transformed malignant cell could express an invasive nature characterized by the appearance of proteolytic enzymes on the cell membrane when the viral genome did not contain those particular genes. Also, in the great majority of animal cancers which occurred spontaneously in nature, no evidence for a virus particle could be found. Indeed, the majority of viruses which could induce

malignancies were active only when paired with cells from species which were not their normal hosts (42). Finally, although diligent efforts have been made, no human malignancy is directly attributable to a virus (42). The most likely candidate would be the Ebstein-Barr virus which may only play a part in the development of Burkitt's lymphoma (43). Therefore, the animal virus models of malignancy are of interest, but dot not appear to be of great importance for drawing correlations to human lesions.

There is no doubt that environmental agents can and do cause malignant tumors (1). But the question here again was how could extremely small molecular weight molecules or radiation alter a normal cell's genetic complement to such an extent that the result was a malignant lesion. The search for normal proteins which had been mutated to renegade states was pursued, but instead of finding new unique proteins which lacked normal regulatory checks and balances, an interesting pattern began to emerge. Knox (44) categorized the levels and types of enzymes present in normal tissues during their embryonic development, and then attempted to place the various malignancies along the spectrum of developing enzymatic activities. He observed that undifferentiated, fast-growing tumors originating from the liver and from mammary gland have almost the same characteristic enzymatic components. Differentiated tumors from liver and mammary gland differ in composition, each tending to resemble the enzymatically different parent tissues. He theorized that a prototypic composition of tumors, from whatever the source, appears to exist. When this composition is diluted by the various enzymes of normal tissues, the products are tumors of lesser neoplastic character which are more differentiated, slower growing, and

less autonomous tumors. Most importantly, he noted the prototypic composition of tumors is very similar but not identical to that of many fetal tissues in both the quantitative pattern of enzymes and the qualitative identities of certain isozymes. It appeared as if a fraction of the cell's genome was acting in the same way in immature and tumorous tissues but differently in adult tissues, i.e. the tumor had dedifferentiated.

Fortunately, considerable evidence is accumulating in favor of models for gene regulation in eukaryotic cells which would support this dedifferentiation theory of malignancy. Experimental results which must be explained by a model are (45): (a) Animal structural genes are in general single copy sequences with an average length of about 1200 nucleotides; (b) Thousands of diverse initiation sites for mRNA synthesis are specified in differentiated cells, indicating a general regulation of transcription; (c) For at least one case, the sea urchin gastrula, over 10^4 specific structural genes are in use, meaning that this number of initiation sites for transcripts which include structural gene sequences must have been activated. This finding probably precludes consideration of any regulatory system that does not involve specific DNA sequence recognition as a fundamental feature; (d) The DNA of eukaryotes displays an ordered interspersion of short repetitive sequences of about 300 nucleotides, and long nonrepetitive sequences of about 1200 nucleotides; (e) This ordered sequence arrangement probably plays some fundamental role in the life process since it has been preserved in quantitatively similar form in sea urchin and amphibian DNA even though the ancestral genomes of these organisms have been evolving along separate lines since the Cambrian period; (f) Lower

organisms such as the lambda phage have early and late functionally related genes which are coordinately transcribed after the appropriate regulation stimulation; (g) Although approximately 70% of the rabbit genome is composed of unique sequence DNA, only 1.8% of this DNA is transcribed in the multipotent cells of the rabbit blastocyst, and the addition of blastocyst RNA to midgestation RNA raises the percentage of hybridizable DNA from 1.8% to 3.2% indicating that there are different genes being transcribed at these periods of development. The progressive restriction concept of gene expression during development is therefore incompatible with the experimental observations. The Britton-Davidson model for animal gene regulation (46) predicts the above observations. Very briefly their model proposes functionally associated batteries of genes which are coordinately activated during the appropriate stage of differentiation. A key feature of their gene regulation model is that the physical basis for coordinated activation of structural genes is the presence of homologous repetitive sequence elements in the vicinity of the initiation sites of genes destined to be activated in concert. Each structural gene in a given battery is preceded by a certain repetitive sequence, the receptor sequence, which is charateristic for that battery. These repetitive sequences serve as recognition and binding sites for sequence-specific regulatory macromolecules termed activators. The term activator is chosen to indicate that the major type of regulator event is expected to be sequence-specific activation of otherwise repressed structural genes (positive control rather than repression). It is considered that nonspecific repression mediated by histones and possibly other chromosomal proteins prevails in all regions of the genome other than those whose activity is specified. By

definition, the diffusible activator molecules must possess the capacity for recognition of specific DNA sequences. Since either protein or RNA could serve this function, both have to be considered as possibilities.

Additional evidence is drawn from clinical evaluation. In recent years several antigens usually present only in fetal tissues have also been found in the sera and tumor tissue of patients with gastrointestinal cancer (47). The carcinoembryonic antigen, alpha-fetoprotein, fetal sulfoglycoprotein antigen, and the carcinoplacental alkaline phosphatase (Regan isoenzyme) are some of the best known. Initially carcinoembryonic antigen and α-fetoprotein were believed to be highly specific diagnostic indicators for colorectal and hepatocellular cancer, respectively (48). Subsequently, carcinoembryonic antigen was shown to be present in other gastrointestinal and nongastrointestinal cancers. The initial reports of elevated serum carcinoembryonic antigen levels in 91% to 97% of colon cancer patients raised hopes that it would serve as a screening test. However, recent data show that carcinoembryonic antigen is increased in less than half of potentially resectable colorectal cancers, and that the previously reported high frequencies are found only in those patients with widespread metastatic cancer (49). In gastric cancer, carcinoembryonic antigen has been found to be abnormal in 73% of the patients with distant metastases. Serum α-fetoprotein, in addition to being frequently positive in hepatocellular carcinoma, has been detected in the sera of some patients with gastric and nongastrointestinal cancer metastatic to the liver (50).

These abbreviated examples suggest strongly the concept that cancer is indeed a developmental disease, that it is not necessary to invoke alterations in the genotype to account for the behavior of malignant

cells. For example, genetic information that is normally and usefully expressed to accomplish mammalian placentation is malignant when expressed in decendents of the embryoblast. The causes of cancer, be they viral, chemical, physical or spontaneous, are interpreted in this view as disrupting mechanisms of gene regulation and integration which results in a dedifferentiated cell with an enzymatic complement tending toward the embryo.

Realizing the tremendous range of differentiation represented by the established mammary tumor lines, it was felt that studies should be conducted on a minimum deviation tumor, and an ascitic line. The R3230 AC mammary adenocarcinoma is the tumor of choice for the former. Hilf et al. (51) originally described this transplantable rat mammary carcinoma of spontaneous origin which does not depend upon estrogen or prolactin for growth, but which would respond to these hormones with distinct alterations in enzymatic activities. Totally specific to the Fisher rat strain, this adenocarcinoma has endogenous levels of α-LA and caseins that can be readily measured. Turkington and Riddle (52) attempted to study how mechanisms which control hormone-dependent genetic expression might be altered in malignant cells. They used explants of normal midpregnant or lactating mammary gland and explants of the R3230 AC carcinoma grown in ovariectomized rats which, after two weeks of tumor growth, were given one mg of estradiol-17β every three days for two weeks prior to tumor removal or oil injections for control animals. Casein synthesis was measured by allowing the explants to incorporate $^{32}PO_4^{3-}$ or ^{14}C-amino-acids for four hours, isolating them by rennin and calcium precipitation, and measuring the labeling using polyacrylamide gel electrophoresis patterns. Explants from normal lactating

glands produced gel patterns of labeled caseins which were qualitatively identical to the estrogen treated R3230 AC gel patterns except that the tumor incorporated only ten percent as much $^{32}PO_4^{3-}$. Control tumors which received no estrogen injections in the host rat nor in the organ culture showed significantly reduced ^{32}P-casein profiles. Enzymatic activities of the two proteins of lactose synthetase, galactosyltransferase and α-lactalbumin, were detectable in homogenates of tumors carried in ovariectomized rats, and estradiol stimulation enhanced both activities. Organ culture experiments done in vitro with 24 hour incubation with estrogen gave similar results to the in vivo experiments. Values for galactosyltransferase activity of the R3230 AC tissue in ovariectomized rats were 6.8% of the lactating rat mammary gland and was raised to 11.6% with estrogen treatment. Similarly, α-LA levels were 2.6% without estrogen and were raised to 23.9% after estrogen stimulation and reflected an increased rate of amino acid incorporation into α-LA. Estradiol treatment does not affect a general increase in synthesis of cytoplasmic proteins in the carcinoma, however. Explants from midpregnant Fisher rat mammary glands incubated for 48 hours in estrogen alone, or in various combinations with insulin, hydrocortisone, or prolactin did not result in a stimulation of casein synthesis or of lactose synthetase activity above the corresponding control value. These observations led them to propose that, since the milk proteins in normal mammary gland tissue were not increased by estrogen alone but were stimulated in the R3230 AC, these malignant cells had acquired a new hormonal responsiveness as a consequence of their neoplastic transformation. In a later paper Turkington and Riddle (53) also demonstrated that a combination of insulin, hydrocortisone and

prolactin could double the level of galactosyltransferase and could increase one and one-third times the level of α-LA in explants from normal virgin rats grown for 48 hours in the hormones. However, the methods they used to assay galactosyltransferase and α-LA utilized incorporation of radioactive galactose from UDP-[U-^{14}C] galactose into lactose and is subject to high backgrounds produced by hydrolysis of UDP-galactose to galactose. In addition, the levels they reported are at the very limit of detection of the incorporation assays. A recent paper by McGuire et al. (54) further confounds the situation by reporting that the R3230 AC tumor is markedly deficient in estrogen receptor content when compared to hormone-dependent tumors or to normal estrogen target tissues.

Estrogen, which circulates in the blood partially bound to serum proteins, is a very lipid soluble steroid hormone which is able to pass through essentially all cell plasma membranes, and is in very rapid equilbrium between the serum and the cytoplasm in cells which do not contain the cytoplasmic estrogen receptor. Found only in tissues which are estrogen sensitive such as the uterus, vagina, or anterior pituitary, this receptor is a large, very elongated prolate ellipsoid of about 227,000 molecular weight which is 18 times as long as it is wide and is composed of two very similar subunits, both of which bind two steroid molecules very tightly (55). Binding of estrogen to the receptor enables both to pass through the nuclear membrane and attach eventually to specific receptor sites on the chromosomes, where it triggers transcription of selected regions of DNA. This recognition of chromosomal regions is directed by the acidic chromatin material rather than the particular DNA base sequence or histones (56).

As is well known, some mammary tumors in both humans and animals depend upon estrogen for continued growth, while others are relatively autonomous. Attempts have been made to link the difference between them with the lack of estrogen receptors in autonomous tumors. McGuire and Julian (57) examined unequivocally proven hormone-dependent and independent mammary tumors induced by dimethylbenzanthracene for the presence of estrogen-receptor in their cytoplasm. They found that the 4S high affinity estradiol-binding moiety was found only in the unequivocally hormone-dependent tumors. They also investigated the R3230 AC and reported that, although the tumor does contain high affinity estradiol binding sites in the cytosol, the concentration of these sites is only 10% compared to the dimethylbenzanthracene induced estrogen dependent tumors, or only 5% compared to normal lactating mammary tissue. It has been rather conclusively shown that the levels of α-LA and galactosyltransferase in the R3230 AC are both sensitive to estrogen administration *in vivo* (58) and that the tumor fails to regress following ovariectomy (54). This paradox of apparent milk protein sensitivity to estrogen, which would require estrogen receptors, and proven autonomous growth, which would theoretically necessitate the absence of estrogen receptors, was explained by a new theory. Keeping in mind that Turkington (9) reported that estradiol *in vitro* had no influence on stimulation or inhibition of R3230 AC cells entering the DNA synthetic phase, McGuire *et al.* (59) cites data suggesting that the effects of estrogen on growth of mammary carcinomas are largely indirect and that the estrogen-dependent tumors are in fact prolactin-dependent. Measurement of prolactin receptor levels in normal lactating gland indicated 3,800 high affinity receptor sites per cell and 2,600 sites per cell for the

R3230 AC tumor. This significant level of prolactin receptors appears to conflict with McGuire's theory of hormone-independent growth of the R3230 AC. The biochemical lesions leading to independent growth must lie distal to the prolactin binding step or alternatively in a function regulated by the estrogen receptor. McGuire feels that there is not necessarily a causal relationship between the relative lack of estrogen receptor and the hormone-independent growth of a tumor. Rather the estrogen receptor may be a useful cell marker for hormone dependence, its absence being a signal that malignant transformation of normal mammary cells has resulted in the deletion of certain components of the normal hormonal regulatory system (59).

Radioimmunoassays

The goals for measurement of a substance are to have the greatest sensitivity with the highest precision, accuracy, and specificity possible. The techniques must be quick and easy to perform and require relatively inexpensive equipment. For many substances, the radioimmunoassay technique is the best choice. Since Yalow and Berson (60) introduced the radioimmunologic method in 1959 for the determination of plasma insulin, it has found applications for many different situations, especially for proteins which do not have a known enzymatic function, such as caseins or β-lactoglobulin. Even for proteins such as α-LA, which have well characterized enzymatic action, there may be great difficulty measuring them by the conventional methods in certain tissues. α-LA participates in lactose formation by lowering the apparent K_m of glucose from 1.4 M to 4 mM so that it becomes a good substrate for galactosyltransferase which combines the substrates UDP-galactose and glucose to

form the disaccharide lactose (3). The proposed enzymatic mechanism is presented in Figure 1. Fitzgerald et al. (61) developed both a spectrophotometric assay for α-LA, which has a realistic limit of detection of two to three μg of α-LA/ml, and a radioactive incorporation assay which has a realistic limit of detection of 0.5 μg of α-LA/ml. The incorporation assay utilizes UDP-[U-^{14}C] galactose and saturating levels of glucose, Mn^{++}, and galactosyltransferase forming labeled lactose. However, many tissues such as lactating mammary gland, kidney, liver, and especially most mammary tumors contain high levels of an enzymatic system which can cleave a variety of nucleotides with the liberation of the respective nucleoside-5'-monophosphates and sugar-phosphates (62) which are then hydrolyzed to the free sugar by broad spectrum phosphatases. Although the pyrophosphatase is membrane associated, it is easily solubilized by homogenization. Geren and Ebner (63) demonstrated that the nucleotide pyrophosphatase can utilize UDP-galactose 100 times more rapidly than the galactosyltransferase. Even using folic acid or 5'-AMP as inhibitors of the hydrolase system, free galactose production can often surpass the production of labeled lactose, resulting in backgrounds for samples that are 70 to 80 percent of the total observed radioactivity in the assay.

The ideal solution to the problem is to utilize an assay which would recognize the unique three-dimensional conformation of α-LA, and this is met by a RIA. In 1974 Beck and Tucker (64) developed a RIA for bovine α-LA which was capable of detecting 25 to 700 ng of α-LA/ml and showed no displacement with one μg of casein, bovine serum albumin or β-lactoglobulin. Two human α-LA RIAs have been reported which both have limits of detection around the one to four ng of α-LA/ml

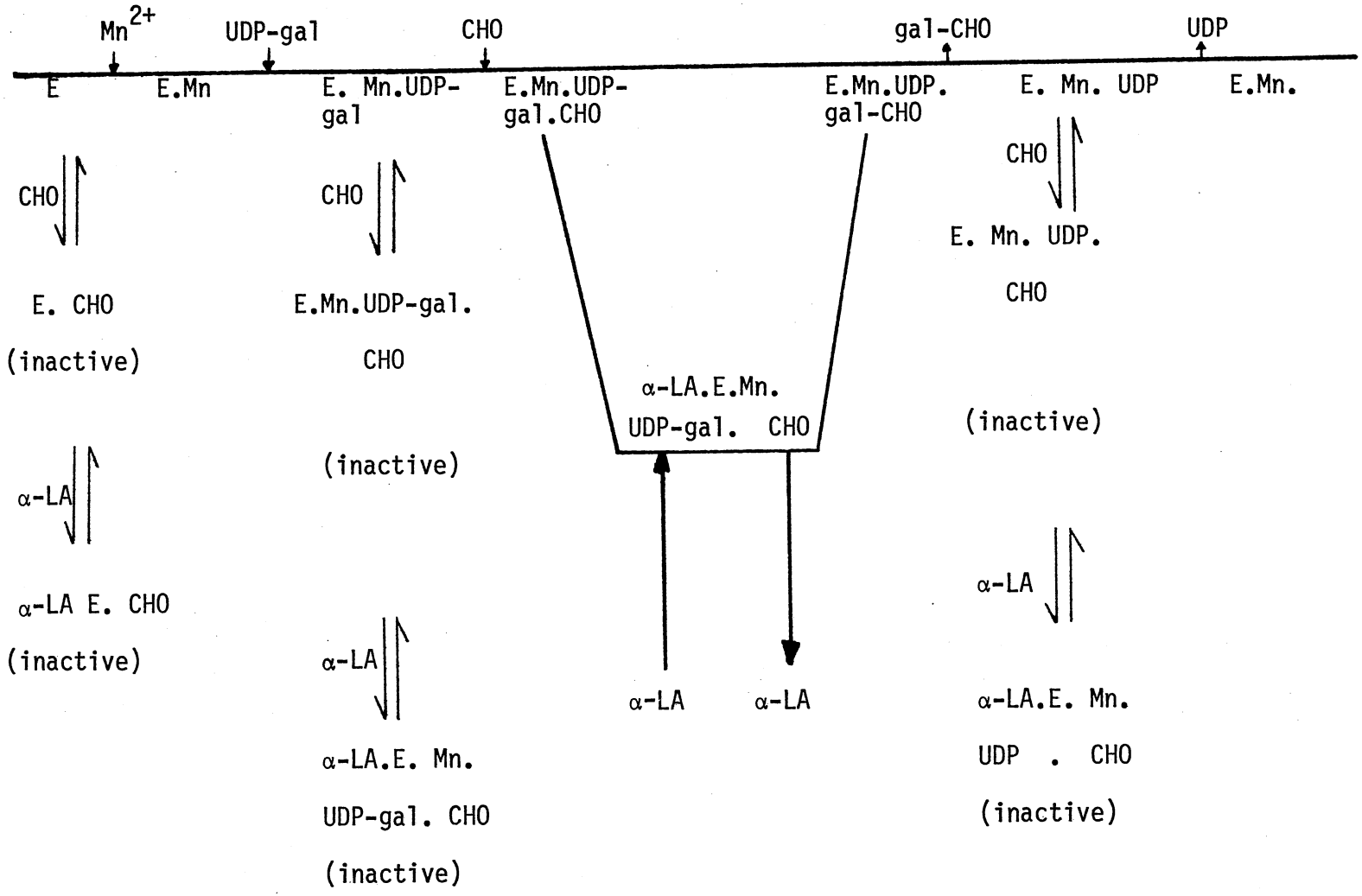

Figure 1. Cleland Representation of the Galactosyltransferase Reaction Sequence

range. Rose and McGrath (65) examined the cross-reactivity of their rabbit anti-human α-LA antibody with α-LA from six animal species by competitive binding in the RIA. None of the α-LAs cross-reacted significantly in the RIA based on the amount of α-LA from the different species required to inhibit 50% of the human α-LA binding (I_{50}). Only 0.24 ng of human α-LA were required as compared with bovine 5.2 μg, rat 9.0 μg, dog 20 μg, and goat 45 μg. Gerbil (25 μg) and pig (50 μg) α-LAs could only achieve 23% displacement, and lysozyme showed no displacement even at 100,000 times molar excess. They isolated a human cell line, designated MCF-7, derived from a pleural effusion of a patient with metastatic breast adenocarcinoma which contains the estradiol binding protein (66). Analysis for α-LA by RIA showed that MCF-7 had 207 ng of α-LA/mg of protein but α-LA could not be induced by incubation of the cell line in media containing 5% calf serum with insulin, hydrocortisone, and prolactin for up to 16 days. Kleinberg (67) used a RIA to determine the levels of α-LA in sera from patients with various diseases. He reported finding α-LA in the sera of normal men and nonlactating females at levels greater than one ng of α-LA/ml in 69% of the males and 46% of the females. The mean of the measurable male values was 5.8 ng of α-LA/ml and the females had a mean of 6.42 ng of α-LA/ml. α-LA was detectable in all lactating sera samples with a mean of 188 ng of α-LA/ml and individual values ranging from 45 to 640 ng of α-LA/ml. α-LA was measured in a group of 35 patients with nonpuerperal galactorrhea. Five patients had undetectable levels and the remaining 30 had a mean level of 55 ng of α-LA/ml, the range being from 2 to 400 ng of α-LA/ml. In a group of 44 patients with metastatic breast carcinoma, five had levels of serum α-LA from one to 12 ng of α-LA/ml. Pro-

lactin serum values were also measured for all the groups and the men had a mean level of 7.13 ng prolactin/ml, normal females 10.9 ng prolactin/ml, nursing mothers 69.3 ng prolactin/ml, galactorrhea 63.4 ng prolactin/ml, and those with breast cancer had 12.4 ng prolactin/ml. When fragments from 19 breast cancers were grown for three days in medium 199 with added insulin and hydrocortisone, only four of the tumors had detectable α-LA concentrations ranging from 0.9 to 3.3 ng of α-LA/ml in their bathing media. Addition of prolactin did not increase α-LA in the media from any of the 19 tumors.

The class of proteins called caseins comprises about 80% of the total milk protein, and are, for the most part, neatly packaged for secretion from the lactating cell in the casein micelle. Caseins are characterized by ester-linked phosphate to specific serine or threonine residues, high proline contents, few or no cysteine residues, and low solubility at pH 4 - 5. However, a clear-cut definition based on these parameters is not possible since some caseins are devoid of phosphate, contain disulfide bonds, have variable proline contents, are soluble at pH 4.5, and are not precipitated at minimum ammonium sulfate concentrations required to precipitate most caseins (68). The caseins of bovine milk have been grouped into four main classes, the α_s-caseins, β-caseins, γ-caseins, and κ-caseins, and their amino acid sequences are known (69). All are characterized by a high content of proline residues randomly distributed throughout the molecules, thus preventing any effective formation of secondary structure such as α-helices. The molecules exhibit zones of high polarity with closely located ionizable groups and other zones that are strongly hydrophobic. In the presence of calcium and phosphate, the caseins have a strong tendency to form

micelles in which the κ-caseins play the key role by stabilizing the other caseins from precipitation. The casein micelle is composed of 63% α_s-casein, 14% κ-casein, 19% β-casein, and 3% γ-casein, with 2.9 gm % Ca^{++}, 0.1 gm % Mg^{++}, 0.8 gm % organic phosphorous, and 1.4 gm % inorganic phosphorous. The discovery of genetic polymorphism in bovine α_s-casein lead to the discovery of four variants whose synthesis is controlled by four allelic autosomal genes with no dominance (70). It is possible that a very large polypeptide chain is first synthesized and then split by limited selective proteolytic cleavage into the individual casein molecules. Certainly, the evidence seems strong that γ-casein and the R, T, and TS fragments are derived from proteolysis of β-casein (71). κ-casein differs markedly from α_s- and β-caseins in that it contains bound carbohydrate and is cross-linked by disulfide groups. One of its outstanding characteristics is a phenylalanine-methionine peptide linkage which is especially sensitive to attack by rennin and other proteases which liberates a glycomacropeptide of 8,000 molecular weight containing the carbohydrate moiety. This hydrolysis renders the remainder of the molecule (para-κ-casein) precipitable by calcium and destroys the ability of κ-casein to stabilize the other caseins against calcium precipitation (72).

Since caseins are under the hormonal control or prolactin, some investigators have chosen to use them as marker proteins for mammary gland function. Hendrich and Franchimont (73) developed a double antibody RIA using rabbit antibodies for human II-caseins and κ-caseins which were isolated by precipitation at low pH, and DEAE ion-exchange chromatography. The caseins were iodinated by the chloramin-T method. The resulting iodinated traces were unstable after eight days and tests

indicated that the labeled II- and κ-caseins existed in polymeric forms. Further analysis has indicated that human casein contains a minimum of six electrophoretically distinguishable caseins, none of which correspond to bovine α_S-casein's migration. Rather, all fall in a narrow range of mobility between bovine γ- and κ-caseins. Amino acid analyses of fractions I-VI are nearly identical with variations seen only in phosphorous content, leading to the view that they represent a single protein with varying levels of phosphorylation. Human κ-caseins are similar to bovine κ-casein (74). In their RIA the smallest detectable amount, defined as the lowest concentration of unlabeled antigen, which when added to a solution containing only labeled antigen and antibody results in significant change in the response variable, was 100 ng/ml with a precision of 8%. Also sera from different patients were examined. Casein was found in the sera of all eleven lactating women with a range of 300 to 2,800 ng casein/ml and an average of 1,070 ng casein/ml. No case was found in the sera of 27 pregnant women. Five percent of 74 normal men and 3% of 36 normal women had levels greater than 10 ng casein/ml. Only one of seven patients with benign breast lesion had detectable concentration of casein: 350 ng casein/ml of serum. Eight of eleven patients, or 72%, with breast cancer prior to any treatment had detectable levels in their serum with a mean value of 283 ± 105 ng/ml. Forty-one patients with breast cancer at Stage I were treated surgically by simple mastectomy with the removal of regional lymph nodes and post-operatively by irradiation and showed no clinical signs of metastases. Seventeen of the forty-one, or 41%, had serum caseins with a mean of 588 ± 160 ng casein/ml. Sixty-one patients with breast cancer had been treated surgically and irradiated post-operatively, but showed evidence

of metastases in the axillary lymph nodes and therefore belonged in Stage II. Thirty-four of the sixty-one, or 50%, had elevated serum casein levels with a mean value of 781 ± 200 ng casein/ml. Casein was found in large quantities in the serum of twenty-one of twenty-six patients presenting with a local recurrence or with distant metastases from their tumor. In these patients, the levels were usually markedly elevated and the mean value of positive cases was 1,235 ± 360 ng casein/ml. In seven of fourteen patients, or 50%, with gastrointestinal neoplasms, casein was found at levels between 120 and 660 ng casein/ml with a mean value of 235 ± 60 ng casein/ml. Seven out of seven patients with lung cancer had elevated casein with a mean value of 342 ± 57 ng casein/ml. Hendrich and Franchimont (73) remark that they have been unable to establish any correlation between the presence of serum casein and the histological nature of the tumor; casein could be found with equal frequency in solid carcinomas in scirrhous cancers, in adenocarcinomas, and in ductal carcinomas. They evoke ectopic exocrine secretion to explain the appearance of caseins in 50% of the gastrointestinal cancers and in 100% of the lung cancer.

Lactoferrin, another protein present in milk, has been studied in the serum of patients with breast cancer (75). Unfortunately this substance is present in sperm and in neutrophile leukocytes and can leak into the serum thus causing false high levels. This undesirable aspect has eliminated the specificity needed for serum analysis.

Immunofluorescence and γ-globulin Receptors

Another method of detecting α-LA in normal and malignant cells is by the indirect immunofluorescent staining technique of Coons (76).

Briefly, this involves incubating a 4 to 6 micron thin section of a tissue with a solution of immune serum containing antibody to the desired molecule, washing unbound γ-globulins off, then flooding the section with a solution containing fluorescein labeled antibodies directed against the original immune γ-globulins. This sandwich technique provides a significant increase in specific fluorescence over the direct labeling technique. Proper controls for this study are, however, absolutely imperative. They include substituting a non-immune serum in place of the immune serum to detect any nonspecific species cross-reactivity or tissue binding of the γ-globulin, and omission of the immune serum to determine any direct binding by the second fluorescent γ-globulin. Bussolati et al. (77) have investigated several human mammary diseases by immunofluorescent methods. Preparing a human casein fraction by merely acid precipitating milk, he prepared rabbit antibodies. Cryostat sections from fresh-frozen tissues were fixed in 95% alcohol for 20 minutes at room temperature and paraffin sections from formalin fixed tissue blocks were de-paraffinized and returned to the water phase. His results indicated most neoplastic cells in medullary and Grade III poorly differentiated carcinomas were negative for casein by immunofluorescence. A thin rim of specific fluorescence at the periphery of the cytoplasm could be seen in some areas of intraductal, in papillary, in mucinous carcinomas, and in some Grade II moderately differentiated infiltrating carcinomas. In intraductal carcinomas with cribriform patterns, casein could be seen lining and sometimes filling intercellular spaces. In Grade I well differentiated carcinomas, casein was present and localized at the inner border of the gland-like structures. In the two cases

examined, cells metastatic to the lymph nodes were also positive, arranged in small clusters in the peripheral sinus. The two cases of adenocarcinoma of the rectum gave negative results.

Examination of mammary and extramammary Paget's disease for casein added weight to the opinion that these clear cells are the result of intraepidermal migration of neoplastic cells from an underlying breast carcinoma, rather than representing a lesion of the nipple epidermis or originating from degenerating melanocytes (78). In 16 cases of Paget's disease of the nipple with underlying ductal carcinomas, intense positive staining was observed in ten cases, while in four cases the staining was definite but weak. In four cases of extramammary Paget's disease of the vulva and anogenital area, one was intense and three were moderate to weak. Control cases of basal cell carcinoma, melanocarcinoma, and intradermal benign nevus gave negative results, but quite significantly the sebaceous and sweat glands in all the tissues gave positive staining.

Using antibodies to bovine β-lactoglobulin, Twarog and Larson (79) examined bovine mammary gland cells in prolonged organ culture and found that they lost the ability to synthesize β-lactoglobulin with time, but that all the secretory epithelial cells showed positive fluorescence for β-lactoglobulin.

In order to determine whether the synthesis of α-LA and casein occurred together within all mammary epithelial cells or within separate cell types, Turkington (80) prepared rabbit antisera to mouse casein component three and Fisher rat α-LA which cross-reacted with mouse α-LA in a non-identity reaction. His results, using a short ethanol-ether fixation on ten day lactating C3H/H_cJ mice, indicated

virtually every alveolar cell was positive for both α-LA and casein. Unfortunately, he did not run the control slide with normal rabbit serum substituting for immune rabbit serum followed by reaction with fluorescent goat antirabbit γ-globulin. As will be shown, rat mammary gland fixed for short periods binds γ-globulins, so his conclusions are not unequivocal.

A very recent report by Kember et al. (81) demonstrates that non-fixed tissue sections from colostrum-forming bovine mammary gland possess highly selective binding activity for secretory immunoglobulin G (I_gG_S). This activity is not found in the bovine post colostrum-forming lactating gland. Inhibition assays showed that I_gG_S binding was blocked by both Fab/c and isolated H-chains but not by bivalent $F(ab)_2$, Fab, and Fc fragments suggesting that the binding region is located within the heavy chains. In addition, I_gG preparations from human, sheep, and rabbit were all equally effective at inhibiting bovine I_gG_S binding, but no inhibition was obtained with nonmammalian turtle and chicken I_gG.

These observations are consistent wtih the known classes of immunoglobulins in mammary secretions, and methods of transmission of immunity from mother to young. Mammals can be classified into at least three groups on the basis of their mode of transmitting immunoglobulins (82). Humans, monkeys, and rabbits (Group I) acquire all of their maternal immunoglobulins in utero and enter the world with a serum level of I_gG equal to that of their mothers. Conversely, ungulates such as cows, horses, pigs, and goats (Group III) derive all maternal immunoglobulins from colostrum during the first few hours after birth. Rats, mice, cats, and dogs (Group II) acquire maternal immunoglobulins both in utero and by way of the colostrum. Only I_gG molecules are transmitted by

animals that receive *in utero* transfer of immunoglobulins. Species that transmit immunoglobulins exclusively *in utero*, Group I, produce colostrum that is predominately composed of I_gA while those that transmit immunoglobulins exclusively via the colostrum, Group III, have a colostrum composed predominately of I_gG and some I_gM and I_gA. Mammals in Group II demonstrate various intermediate compositions with high levels of both I_gG and I_gA but low I_gM. It is interesting to note that Group I animals such as man have no immunoglobulin absorption by the gut of the newborn. The proteolytically resistant I_gA molecules are thought to provide essential resistance to intestinal bacterial or viral infections for the newborn. The Group III mammals have extensive absorption by the newborn's gut for up to 48 hours with some species showing selective absorption of I_gG. Rats and mice from Group II have moderate rates of gut absorption and are selective for I_gG but absorption continues for 19 days post parturition. These observations may indicate which immunoglobulin subclasses should or should not bind to colostrum or mature lactating mammary epithelial cells.

CHAPTER III

DEVELOPMENT OF RADIOIMMUNOASSAYS

Isolation of Rat and Human α-Lactalbumin

Materials and Reagents

Rat milk was obtained from lactating Fisher rats throughout their lactational period by gentle aspiration of the nipples after anesthetization by intramuscular injection of pentabarbital (0.1 ml/150 gm) and induction of milk release with intramuscular injection of oxytoycin (0.5 ml/150 gm). Human breast milk was a gift from the Oklahoma Medical Research Foundation. Pyruvate kinase (Type I from rabbit muscle in ammonium sulfate suspension containing lactic dehydrogenase), glycine, phosphoenolypyruvate, NADH, Tris, bovine serum albumin, and bovine γ-globulin fraction II were purchased from Sigma Chemical Company. Acrylamide gel electrophoresis reagents and Bio-Gel P were purchased from Bio-Rad. DEAE-cellulose was purchased from Whatman. Small bore 4,000 to 6,000 molecular weight cutoff dialysis tybing was purchased from Spectrapore.

Methods

Spectrophotometric Assay for α-Lactalbumin. α-LA was assayed in the presence of saturating amounts of galactosyltransferase, Mn^{++}, glucose, and UDP-galactose according to the method of Fitzgerald et al.

(61). Enzymatic activity was assayed spectrophotometrically at 340 nm by coupling UDP formation to NADH oxidation by adding phosphoenolpyruvate and pyruvate kinase.

Gel Filtration and Ion Exchange Chromatography. Molecular weight gel filtration was performed at 4° C on samples dissolved in 100 mM KCl, 20 mM Tris, pH 7.4 which was the column buffer. A 5 cm x 100 cm column was packed by the slurry technique with P-150, 100 to 200 mesh, which had been swollen, degassed and had the fines removed according to the Bio-Rad manual (83). Using a pressure head of 30 cm, approximately 400 mg of protein in a volume of 15 ml were applied and washed into the column. Five ml fractions were collected with a seven s/drop flow rate. Dextran blue was never mixed with the samples to eliminate nonspecific binding to the α-LA. DEAE-32 was generated by the procedure recommended by the Whatman manual (84), and packed in a 2.5 cm x 25 cm column and washed with 20 mM Tris, pH of 7.4 until the effluent had a conductivity of less than one m mho. Samples to be applied were dialyzed in 6,000 molecular weight cutoff dialysis tubing against 20 mM Tris, pH 7.4 until its conductivity was also less than one m mho. After the sample was washed into the column at 4° C, a two liter total volumn linear gradient of 0 to 300 mM KCl, 20 mM Tris, pH 7.4 was passed through the column at a flow rate of six seconds per drop and five ml fractions were collected.

Acrylamide Gel Electrophoresis. Standard discontinuous gels, 10 cm x 0.5 cm, at pH 9.5 were prepared according to the method of Davis (85). Sample solutions were dialyzed overnight against the upper gel buffer, 5 µl of 0.005% bromophenol blue in water was added as a

tracking dye and solid sucrose added until the protein solution was more dense than the upper reservoir buffer. After layering the sample onto the stacking gel, the gels were run at 7 m amps/gel at room temperature until the tracking dye was near the end of the gel, at which time they were removed from the tubes. The tracking dye was marked by a wire and the gels were placed in 7% acetic acid (v/v) and 0.007% (w/v) Coomassie Blue overnight to stain the protein bands. Destaining was by diffusion against 7% acetic acid. Two sodium dodecyl sulfate systems were utilized. The standard non-discontinuous system of Weber and Osborn (86) was followed exactly with a 30 minute pre-electrophoresis step. The Neville (87), or discontinuous gel system, was not modified and sample preparation and current levels for both systems were as described in the respective papers. After marking the dye front, sodium dodecyl sulfate was removed from the gels by extraction in 40% methanol and 7% acetic acid (v/v), and stained and destained as for the discontinuous gels. Final gel patterns were scanned at 560 nm using the Gilford Model 240 spectrophotometer with the linear transport unit.

Acrylamide gels were sliced with the Bio-Rad gel slicer Model 190 into 2 mm thick sections. Protein was extracted from the gel slices by macerating the gel in 0.5 ml of 0.1 M KCl, 20 mM Tris, pH 7.4 followed by freezing and thawing.

Results

McFarland (88) attempted to isolate α-LA from Fisher rat milk using the procedure of Schmidt *et al.* (89). Purity of the final material was determined by gel electrophoresis on short (5.5 cm x 0.5 cm) 7% acryla-

mide gels prepared by the Davis procedure but cast without the stacking gel. Detection of protein by staining with Aniline Blue Black revealed at least three major bands, two of which had very similar migration coefficients of about 0.5 and one band which had a low migration coefficient of about 0.1. Spectrophotometric analysis of macerated and extracted gel slices indicated that both the fast and slow migrating bands contained α-LA activity. This observation led to the proposal that rat α-LA existed in two forms. However, the material was not analyzed by sodium dodecyl sulfate gel electrophoresis.

An attempt was made to reproduce McFarland's observations using the same isolation procedure. The final material was analyzed by discontinuous gel electrophoresis according to the procedure of Davis (85) on long (10 cm x 0.5 cm) 7% acrylamide gels at pH 9.5. Detection of protein by staining with the more sensitive Coomassie Blue produced the gel profile shown in Figure 2. Spectrophotometric analysis of macerated and extracted gel slices indicated that only one protein band contained α-LA. Sodium dodecyl sulfate gel electrophoresis of the material isolated by the Schmidt (89) procedure indicated the molecular weights of the two major protein bands were about 20,000 and 60,000. An attempt was made to separate the α-LA from the higher molecular weight contaminants by applying the sample at room temperature to a 100 cm x 1 cm column of P-100, 50 to 100 mesh, equilibrated and eluted with 0.1 M KCl, 20 mM Tris, pH 7.4. One ml fractions were collected, and the protein profile established by absorbance at 280 nm. Fractions were assayed for α-LA by the spectrophotometric procedure. As shown in Figure 3, the α-LA activity was effectively resolved from the higher molecular weight contaminating proteins.

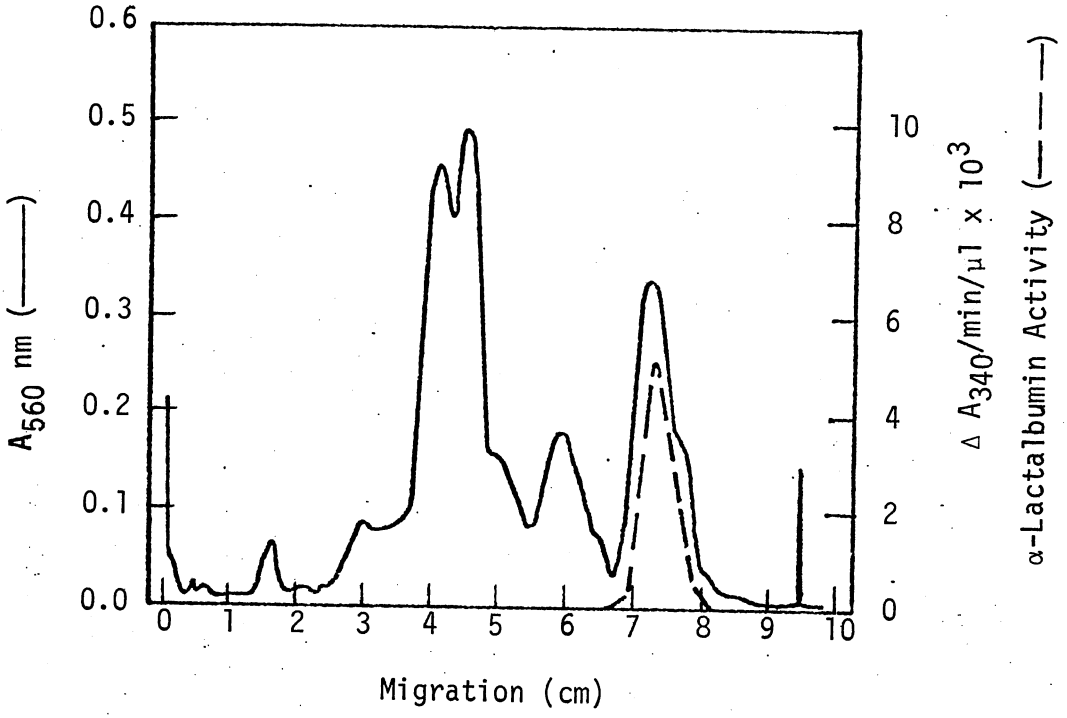

Figure 2. Discontinuous Gel Electrophoresis of Rat α-Lactalbumin Purified by the McFarland Procedure

Rat α-LA purified by the McFarland procedure (88) was applied to 7.5 percent, 10 cm x 0.5 cm discontinuous polyacrylamide gels at pH 9.5, stained, and scanned at 560 nm. Gels were cut into 2 mm slices, macerated, and extracted with 0.5 ml of 0.1 M KCl, 20 mM Tris, pH 7.4 by repeated freezing and thawing. The extracted solutions were assayed for α-LA by the spectrophotometric method at 340 nm (Methods).

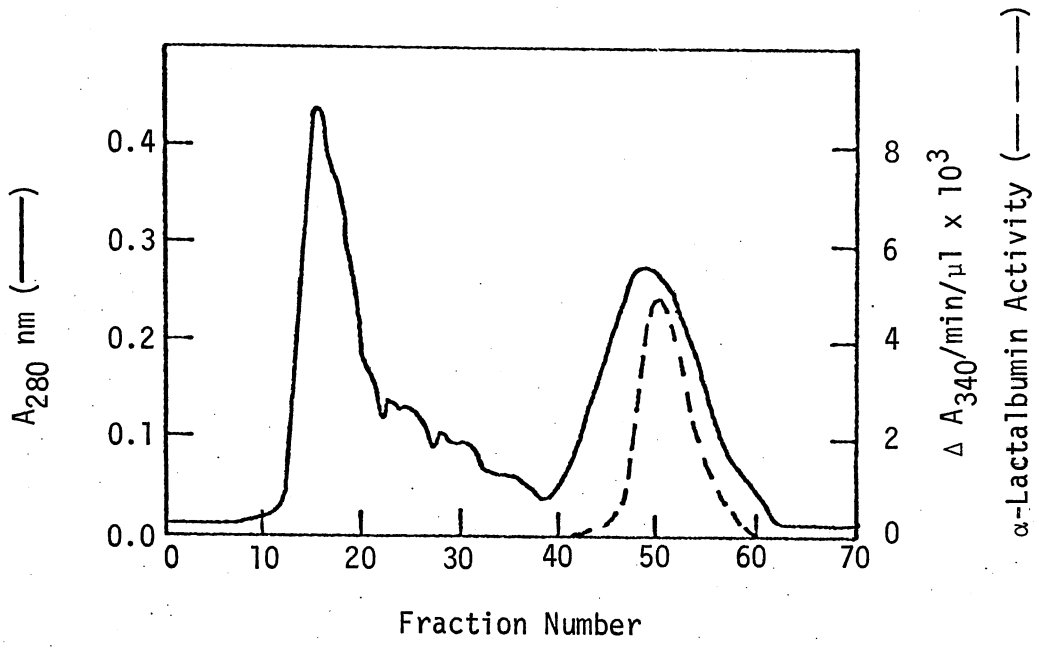

Figure 3. Elution Profile of Whey Proteins from Rat Milk on Bio-Gel P-100

The whey fraction of rat milk was prepared by fractionation at 40% and 80% saturation of ammonium sulfate and centrifugation at 100,000 x g for one hour, and was applied to a 100 cm x 1 cm column of P-100 equilibrated and eluted with 0.1 M KCl, 20 mM Tris, pH 7.4. One ml fractions were collected and the protein profile determined by absorbance at 280 nm. Fractions were assayed for α-LA by the spectrophotometric method at 340 nm (Methods).

An attempt was made to isolate human α-LA based on the experience gained from the isolation of rat α-LA. The final procedure, which was performed at 4° C was as follows. Human Negro breast milk was received frozen and was slowly thawed. The milk was centrifuged at 15,000 x g for 30 minutes in a RC-2B Sorvall centrifuge using the SS-34 head. The cream was removed by filtering the centrifuged solution through Whatman Shark Skin filter paper. The skimmed milk was centrifuged at 100,000 x g for one hour in a Beckman L5-65 ultracentrifuge using the SW27 head to sediment the casein micelles. The supernatant solution was made 40% (0.226 g/ml) saturated with ammonium sulfate and centrifuged at 30,000 x g for 30 minutes in the RC-2B centrifuge with the SS-34 head. The supernatant solution was made 80% (0.516 g/ml) saturated with ammonium sulfate and centrifuged as before. The pellet was resuspended with 0.1 M KCl, 20 mM Tris, pH 7.4 at a protein level of 30 mg/ml. Approximately 0.5 grams of protein (20 ml) was applied to a 5 cm x 100 cm column of P-150, 100 to 200 mesh, equilibrated and eluted with 0.1 M KCl, 20 mM Tris, pH 7.4. A pressure head of 30 cm produced a 7 s/drop flow rate and 5 ml fractions were collected. The protein profile was determined by absorbance at 280 nm and α-LA activity was located by the spectrophotometric assay. As shown in Figure 4, the α-LA activity was cleanly separated from the higher molecular weight proteins. Fractions 110 to 140 were pooled and dialyzed in the 6,000 molecular weight cut-off dialysis tubing against 20 mM Tris, pH 7.4 until the sample's conductivity was identical to the conductivity of 20 mM Tris, pH 7.4. The sample was then applied on a 2.5 cm x 25 cm column of DEAE-32 cellulose equilibrated with 20 mM Tris, pH 7.4. The column was washed with 100 ml of 20 mM Tris, pH 7.4 and a 2 liter total volume linear gradient of

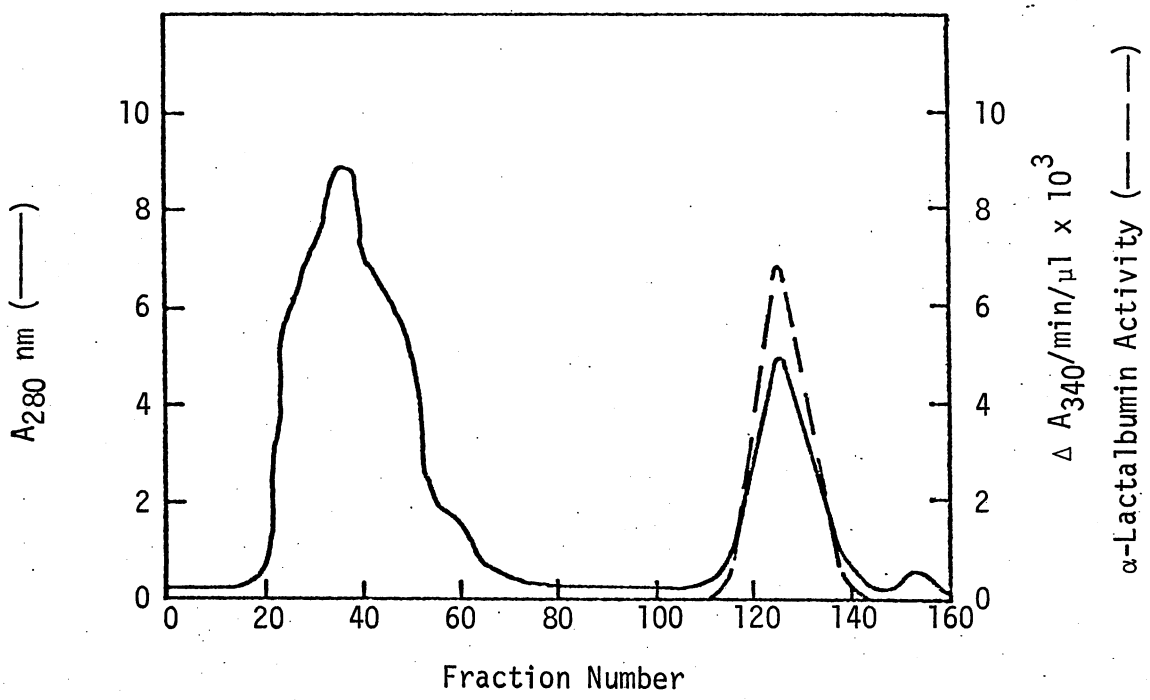

Figure 4. Elution Profile of Whey Proteins from Human Milk on Bio-Gel P-150

The whey fraction of human milk was prepared by fractionation at 40% and 80% saturation of ammonium sulfate and centrifugation at 100,000 x g for one hour, was applied to a 100 cm x 5 cm column of P-150 equilbrated and eluted with 0.1 M KCl, 20 mM Tris, pH 7.4. Five ml fractions were collected and the protein profile determined by absorbance at 280 nm. Fractions were assayed for α-LA by the spectrophotometric method at 340 nm (Methods).

0 to 300 mM KCl, 20 mM Tris, pH 7.4 was passed through the column at a flow rate of 6 s/drop. Three ml fractions were collected. The protein profile and α-LA activity were determined as described before. As shown in Figure 5, the α-LA activity was sharply eluted and coincided with the major protein peak. A small contaminant was resolved from the α-LA peak. Fractions 90 to 100 were pooled, extensively dialyzed in 6,000 molecular weight cutoff tubing against deionized water, and lyopholyzed for storage at -20° C.

Rat α-LA was isolated by C. Brown (90) using the identical procedure, except that the α-LA peak from the DEAE-32 column was reapplied to the P-150 column for an additional purification step.

The human α-LA was analyzed by the Davis (85) discontinuous gel system and the Weber and Osborn (86) sodium dodecyl sulfate gel system. As shown in Figure 6, no contamination was detectable in the gels of either system even when 100 μg of protein were applied to the gels.

Generation of Anti-α-Lactalbumin Serum

Materials and Reagents

The rat and human α-LA samples purified above were used as antigen sources. Freund's complete and incomplete adjuvants and agar were from Difco. α-LAs from various species were prepared by Dr. D. Schmidt except for bovine α-LA which was from Sigma. Chemicals for immunoelectrophoresis and instruments were from Gelman. Balanced salt solution was 0.14 M NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 0.17 mM NaH_2PO_4 at pH 7.2. The Polytron Model P-10 homogenizer was from Brinkman.

Methods

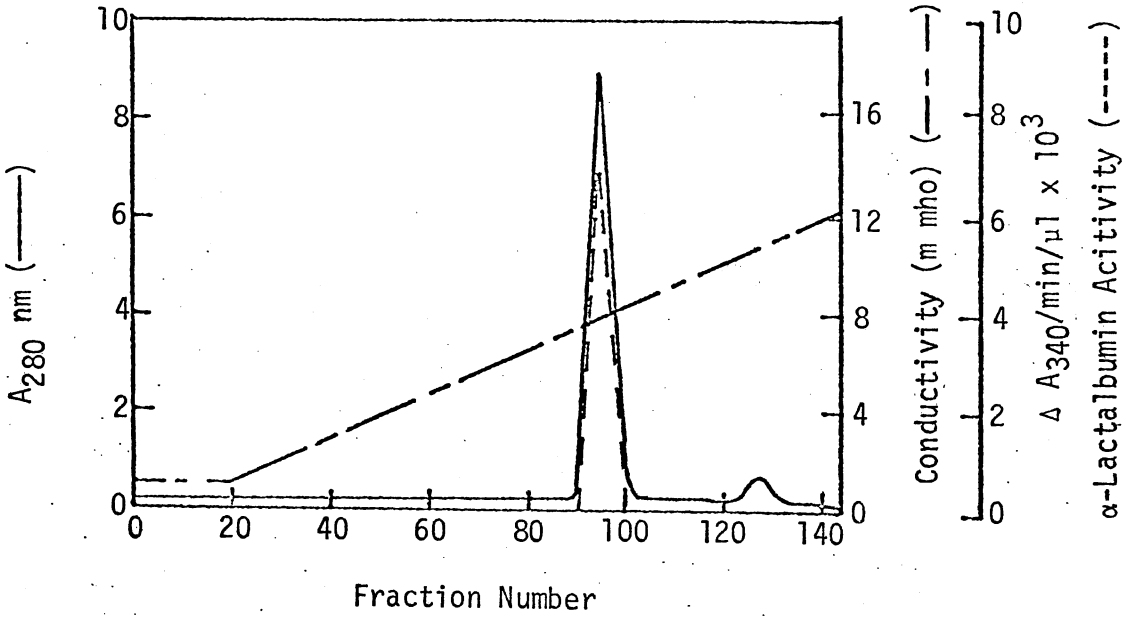

Figure 5. Elution Profile of Human α-Lactalbumin from P-150 Column on DEAE-32 Cellulose

The pooled α-LA fraction from P-150 column was dialyzed extensively against 20 mM Tris, pH 7.4 and applied to a 25 cm x 2.5 cm column of DEAE-32 equilibrated in 20 mM Tris, pH 7.4. The column was washed with 100 ml of 20 mM Tris, pH 7.4 and a two liter total volumn linear gradient of 0 to 300 mM KCl, 20 mM Tris, pH 7.4 was passed through the column and 3 ml fractions were collected. Protein profile was determined by absorbance at 280 nm. Fractions were assayed for α-LA by the spectrophotometric method at 340 nm. The KCl gradient was measured by a conductivity meter (Methods).

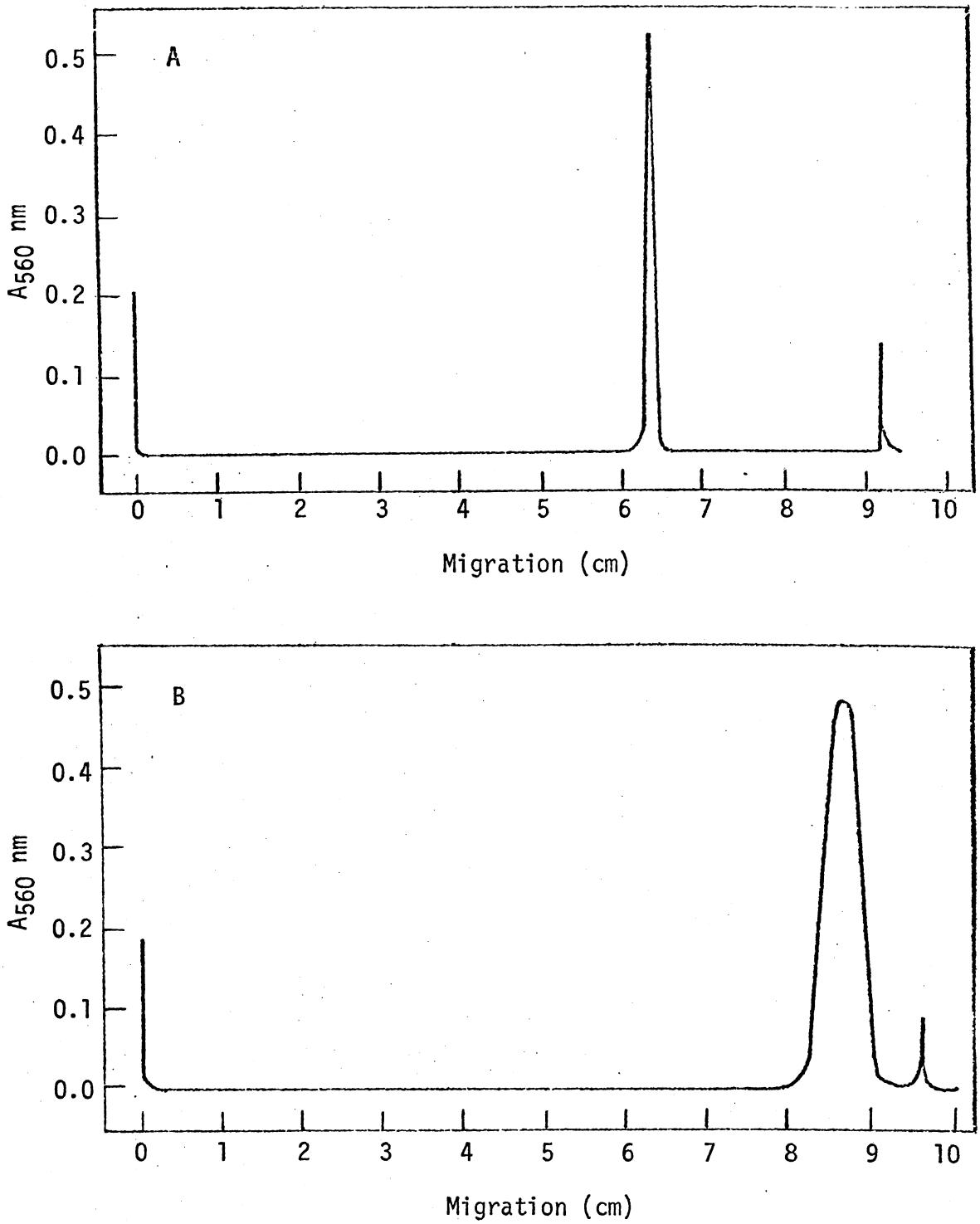

Figure 6. Acrylamide Gel Electrophoresis of Purified Human α-Lactalbumin

Human α-LA (100 μg) from the DEAE-32 column was applied to 10 cm x 0.5 cm gels prepared by (A) Davis (85) procedure, and (B) Weber and Osborn (86) procedure, stained with Coomassie Blue, and scanned at 560 nm (Methods).

Adult white rabbits were injected in two places subcutaneously on the back with a total of 4 mg of human or rat α-LA dissolved in 0.5 ml of balanced salt solution and mixed with 6 ml of Freund's complete adjuvant and then homogenized with a Polytron set at 5.4 for two minutes at 4° C. After two weeks, 4 mg more of α-LA in 4 ml of Freund's incomplete adjuvant were prepared as before and injected subcutaneously on the back. Blood was drawn from the ear vein or by heart puncture and immediately centrifuged at 5,000 x g for 20 minutes at room temperature to separate the serum. Immunoelectrophoresis was done according to the method in the Gelman manual (91). Double diffusion plates were 1% agar in 0.1 M KCl, 20 mM Tris, pH 7.0 and were developed at 4° C, 25° C, or 37° C, and then photographed. All antigen solutions were 0.5 mg/ml in balanced salt solution and antisera were undiluted.

Results

Sera collected three weeks after injection of rat or human α-LA showed weak precipitin lines in double diffusion tests against the antigens. Five weeks after injection, strong bands were seen against rat or human α-LA. Cross-reactivity to α-LA from other species was investigated using the double diffusion technique with antisera drawn at various times after injection of α-LA. As shown in Figure 7, antirat α-LA sera consistently had only weak cross-reactivity to human α-LA which demonstrated non-identity. However, antihuman α-LA sera had cross-reactivity to rat, bovine, goat, and pig α-LA five weeks after injection which was not detectable three weeks after injection. This observation indicates that human α-LA is capable of eliciting a time-dependent cross-reactivity spectrum in which possibly less antigenic determinant

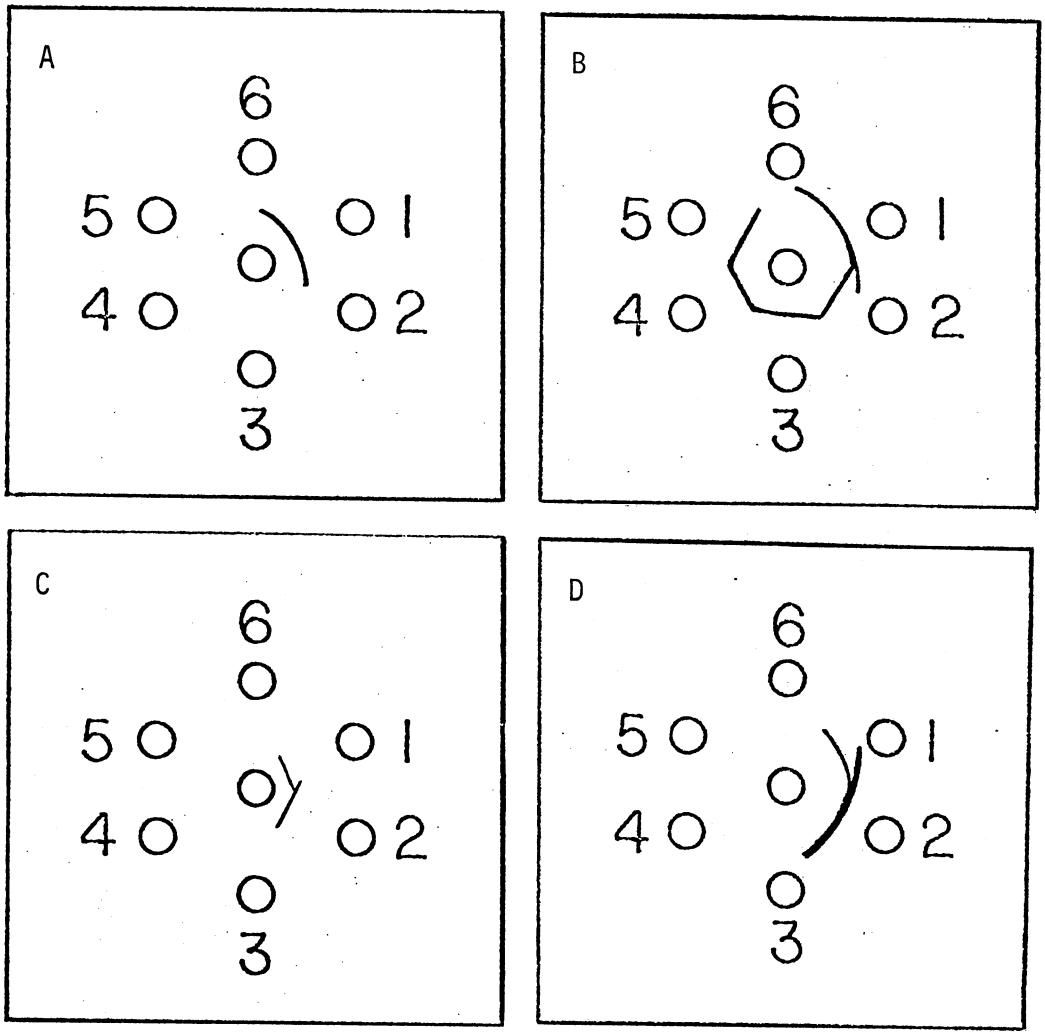

Figure 7. Cross-Reactivity of Anti-α-Lactalbumin Sera

Rabbits were injected subcutaneously with α-LA in Freund's adjuvant and sera were collected after various times of immunization and tested for cross-reactivity to α-LA from other species in double diffusion plates of 1% agar, 0.1 M KCl, 20 mM Tris, pH 7 at 25° C. Wells contained (1) human α-LA, (2) rat α-LA, (3) bovine α-LA, (4) pig α-LA, (5) goat α-LA, and (6) bovine serum albumin, all at 0.5 mg/ml. Panel (A) and (B) contained antihuman α-LA serum drawn at three weeks and five weeks respectively in the center wells. Panel (C) and (D) contained antirat α-LA serum drawn at three weeks and five weeks respectively in the center wells (Methods).

sites, presumably preserved through the different species' α-LA structures, eventually become expressed. This may account for the apparently conflicting claims for cross-reactivity of antihuman α-LA antibodies (65, 92). In double diffusion tests, both antisera had single precipitin bands against their whey fraction, and no bands were seen against egg white lysozyme, rat serum albumin, bovine serum albumin, bovine casein fraction, or against male rat or human sera.

Immunoelectrophoresis of both rat and human α-LA was performed with their corresponding antisera and the results are shown in Figure 8. They indicate that there is a single precipitin arc against human α-LA, but two arcs for rat α-LA, which is a reflection of the different electrophoretic mobilities of the charge isomers for rat α-LA (90). The same patterns were seen when reacted against their electrophoretically migrated whey fractions.

Determination of the Optimal Conditions for the Radioimmunoassay for α-Lactalbumin

Materials and Reagents

GA-6 and GA-8 filters were purchased from Gelman. The filter manifold unit Model P-10 was purchased from the Bradley Company. Polyethylene glycol with average molecular weight 18,000 was obtained from Matheson. Carrier free ^{125}I was purchased from New England Nuclear. Solutions of monoiodochloride, lactoperoxidase, and chloramine-T were supplied by G. Polack and L. Walker respectively. Dowex-1-Cl, 50 to 100 mesh, was purchased from Bio-Rad. Counting ^{125}I was performed on Packard's Model 5320 instrument according to the instruction manual.

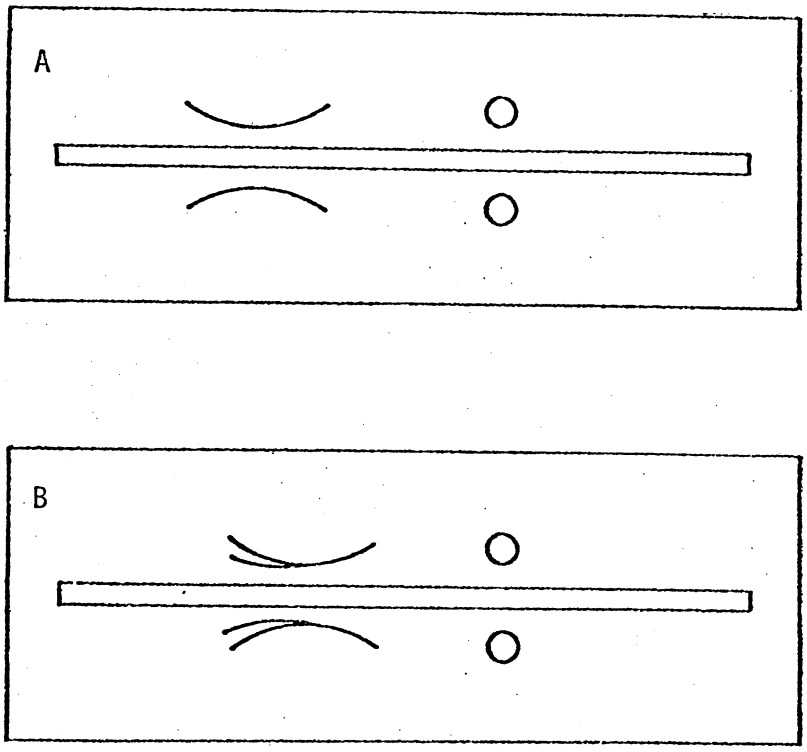

Figure 8. Immunoelectrophoresis Against Anti-α-Lactalbumin Sera

Immunoelectrophoresis of samples containing α-LA was performed using antisera made in response to the purified human and rat α-LA. Panel (A) top well is purified human α-LA at 0.5 mg/ml, bottom well is the whey fraction of human milk, and the trough contains antihuman α-LA serum. Panel (B) top well is purified rat α-LA at 0.5 mg/ml, bottom well is the whey fraction of rat milk, and the trough contains antirat α-LA serum. Electrophoresis was performed until the tracking dye was at the end of the trough. The troughs were then filled with their antiserum and incubated at 25° C for 12 hours (Methods).

Methods

Iodination of α-LA was accomplished by three different techniques, i.e. monoiodochloride (93), chloramine-T (94), and lactoperoxidase (95). Separation of the free and covalently bound ^{125}I was accomplished by dialysis against 1% KI in H_2O and binding of the free ^{125}I on Dowex-1-Cl. Purified ^{125}I-α-LA was diluted with either 0.1 M KCl, 20 mM Tris, pH 7.4, or the same solution with 0.1% bovine serum albumin (w/v) added and stored at 4° C.

Results

Separating free ^{125}I-α-LA from the antibody bound ^{125}I-α-LA was achieved by precipitating the antibody ^{125}I-α-LA complex with polyethylene glycol. To demonstrate that the free ^{125}I-α-LA molecules are not precipitated at 10% (w/v) polyethylene glycol while the antibody molecules are precipitated, the following experiment was performed. One hundred µl of rat ^{125}I-α-LA, containing 40,000 dpm with a specific activity of 80 µCi/µg, in 0.1 M KCl, 20 mM Tris, pH 7.4 with 0.1% bovine serum albumin, was made to various percentages of polyethylene glycol by the addition of 25% polyethylene glycol solution in water. The samples were thoroughly mixed and placed in an ice bath for 10 minutes, then filtered through GA-8 filters using the Bradley filter manifold. The filters were washed twice with 2 ml of polyethylene glycol of the same concentration used for that particular test point. The filters were placed in one inch aluminum foil squares and counted in the Packard gamma counter. Quench corrections were performed (96) and radioactivity measurements were based on disintegrations per minute. As shown in Figure 9, some ^{125}I-α-LA is trapped by the filters with no polyethylene

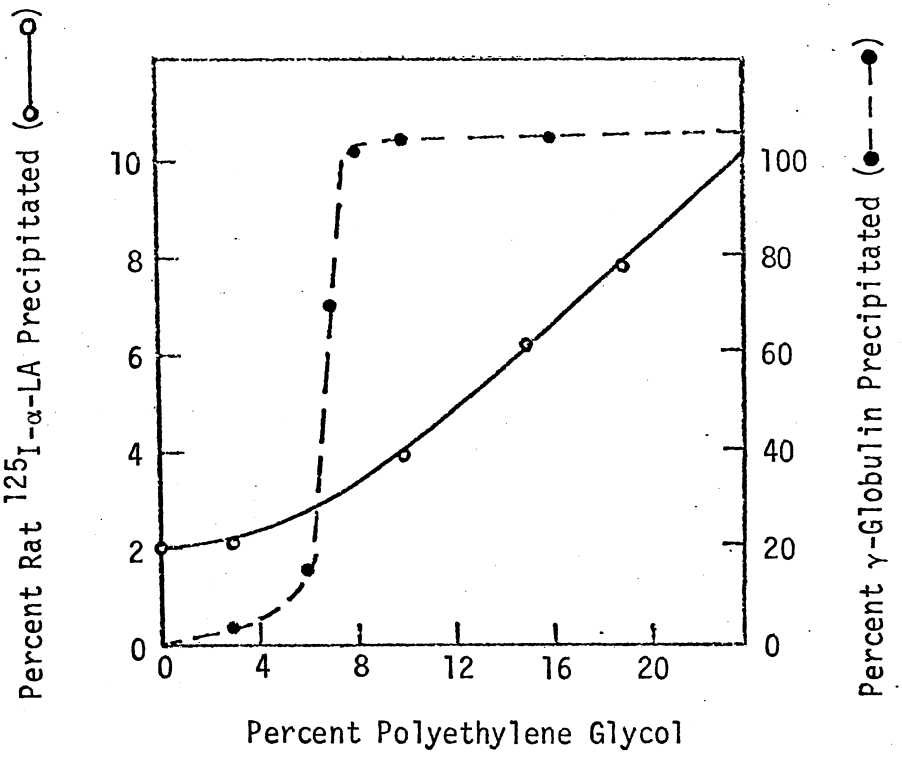

Figure 9. Effect of Polyethylene Glycol on the Precipitation of Rat ^{125}I-α-Lactalbumin and Bovine γ-Globulins

One hundred μl samples of rat ^{125}I-α-LA containing 40,000 dpm were made to various percentages of polyethylene glycol, filtered through GA-8 filters, and the precipitated radioactivity measured in a gamma counter. Samples of bovine γ-globulins at 1 mg/ml were made to various percentages of polyethylene glycol and centrifuged at 30,000 x g for 20 minutes. The absorbance of the supernatant solutions was measured at 280 nm using the corresponding polyethylene glycol solution as the reference blank (Results).

glycol present. At 10% polyethylene glycol, only 1.5% of the total ^{125}I-α-LA molecules are retained on the filter above background trapping.

To determine the concentration of polyethylene glycol that would quantitatively precipitate the antibody molecules, the experiment shown in Figure 9 was performed. One ml samples of a 1 mg/ml bovine γ-globulin solution in 0.1 M KCl, 20 mM Tris, pH 7.4 were made to various percentages of polyethylene glycol by the addition of 25% polyethylene glycol in water. The samples were placed in an ice bath for 10 minutes then centrifuged at 30,000 x g for 20 minutes at 4° C. Absorbance at 280 nm of each supernatant solution was determined by referencing the solution against the appropriate percent polyethylene glycol solution. At 10% polyethylene glycol concentration 99% of the antibody molecules are precipitated. These results indicate that at a 10% concentration of polyethylene glycol, the antibody molecules are quantitatively precipitated while the ^{125}I-α-LA molecules essentially remain in solution.

The principle of competitive binding assays, such as a RIA, requires that the antibody always be in a constant limiting amount. Therefore, the RIAs were performed using a serum dilution which could only bind 50% of the available ^{125}I-α-LA molecules. An antiserum dilution was sought which would bind 50% of the total 40,000 dpm of rat ^{125}I-α-LA. Antirat α-LA serum was diluted with 0.1 M KCl, 20 mM Tris, pH 7.4 and 100 µl were added to 100 µl of rat ^{125}I-α-LA (specific activity 80 µCi/µg) containing 40,000 dpm in 0.1 M KCl, 20 mM Tris, pH 7.4. The mixture was incubated 24 hours at 4° C. Then 500 µl of 1 mg/ml bovine γ-globulin in 0.1 M KCl, 20 mM Tris, pH 7.4 were added as carrier γ-globulin, and 500 µl of 25% polyethylene glycol in water were added.

The solution was thoroughly mixed and placed in an ice bath for 10 minutes, then filtered through GA-8 filters and washed twice with 2 ml of 8% polyethylene glycol. Controls for each point were done by omitting the antirat α-LA antibodies from the reaction mixtures. The filters were placed in one inch squares of aluminum foil and counted in a Packard gamma counter. The results shown in Figure 10 indicate that a serum dilution of 1 to 10,000 binds about 50% of the total rat ^{125}I-α-LA molecules added.

The RIA is based upon the ability of the antibody molecules to recognize the α-LA molecules. It is important to determine what factors can influence this binding. The effect of pH was investigated by incubating antirat α-LA antibody and rat ^{125}I-α-LA with different pH buffers. One hundred μl of antirat α-LA antibody solution diluted 1:8000 with 0.1 M KCl, 5 mM Tris, pH 7.0 were incubated with 100 μl of rat ^{125}I-α-LA (specific activity 80 μCi/μg) containing 40,000 dpm in 0.1 M KCl, 5 mM Tris, pH 7.0. To this solution were added 100 μl of the different pH buffers. The different buffers were all 0.1 M. Phosphate was used as the buffer at pH 2, pH 7, and pH 12. Citrate was the buffer at pH 4.5 and pH 5.8. Glycine was the buffer at pH 9.5. The samples were incubated for 24 hours at 4° C. Then 0.5 ml of 1 mg/ml bovine γ-globulin in 0.1 M KCl, 5 mM Tris, pH 7.0 and 0.5 ml of 25% polyethylene glycol in water were added. The solution was vigorously mixed and placed in an ice bath for 10 minutes then filtered through GA-8 filters and washed twice with 2 ml of 8% polyethylene glycol. The filters were placed in aluminum foil squares and counted in a Packard gamma counter. Controls for each pH point were performed by omitting antirat α-LA antibodies from the reaction mixtures. As shown in

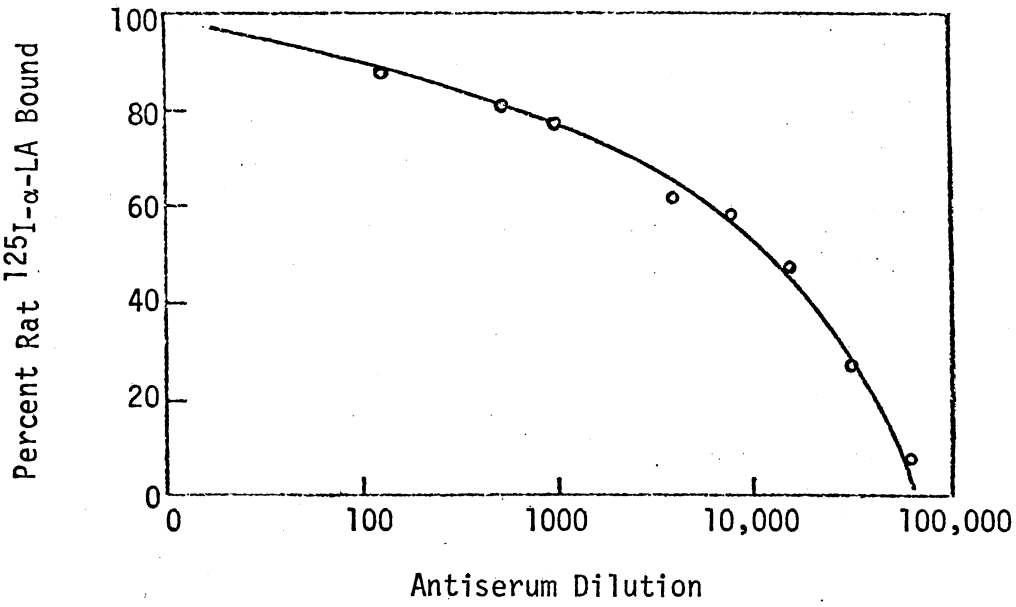

Figure 10. Determination of Optimal Antiserum Dilution

Rabbit antirat α-LA antiserum was diluted with the standard RIA buffer and 100 µl were incubated with 100 µl of rat ^{125}I-α-LA at 4° C for 24 hours. The polyethylene glycol precipitation technique was used to separate free and antibody bound rat ^{125}I-α-LA. One hundred percent represents 40,000 dpm of rat ^{125}I-α-LA (Results).

Figure 11, the optimal binding will occur between pH 7 and pH 9. Because the curve is slightly shifted towards basic conditions, 20 mM Tris, pH 7.4 was used as the buffering agent.

The effect of ionic strength on antigen-antibody binding was investigated. As before, 100 µl of antirat α-LA serum diluted 1:8000 with 5 mM Tris, pH 7.4 were added to 100 µl of rat ^{125}I-α-LA (specific activity 80 µCi/µg) containing 40,000 dpm in 5 mM Tris, pH 7.4. To this solution were added 100 µl of solutions of KCl at different molar concentrations. Controls for each ionic strength point were done by omitting the antirat α-LA antibodies from the reaction mixtures. The samples were incubated, precipitated, washed, and counted as before. Figure 12 shows that optimal binding occurs around 0.1 M KCl, and that binding is dramatically reduced in very low ionic strength solutions. Binding also drops to about 80% maximum and then levels off above 0.4 M KCl. Based on these results, the standard buffer for the RIAs contains 0.1 M KCl.

Frequently, it is necessary to assay samples that have high protein concentrations. As pointed out by Viol et al. (97), most RIAs can be inhibited nonspecifically by high protein levels resulting in false positive values. The effect of high protein levels on the α-LA RIAs was investigated by adding bovine serum albumin. Double diffusion tests indicated no cross-reactivity of either antiserum to bovine albumin. One hundred µl of antirat α-LA serum diluted 1:8000 in 0.1 M KCl, 20 mM Tris, pH 7.4 were added to 100 µl of rat ^{125}I-α-LA (specific activity 80 µCi/µg) containing 40,000 dpm in 0.1 M KCl, 20 mM Tris, pH 7.4. To this solution were added 100 µl of bovine albumin in 0.1 M KCl, 20 mM Tris, pH 7.4 at different protein levels. Controls

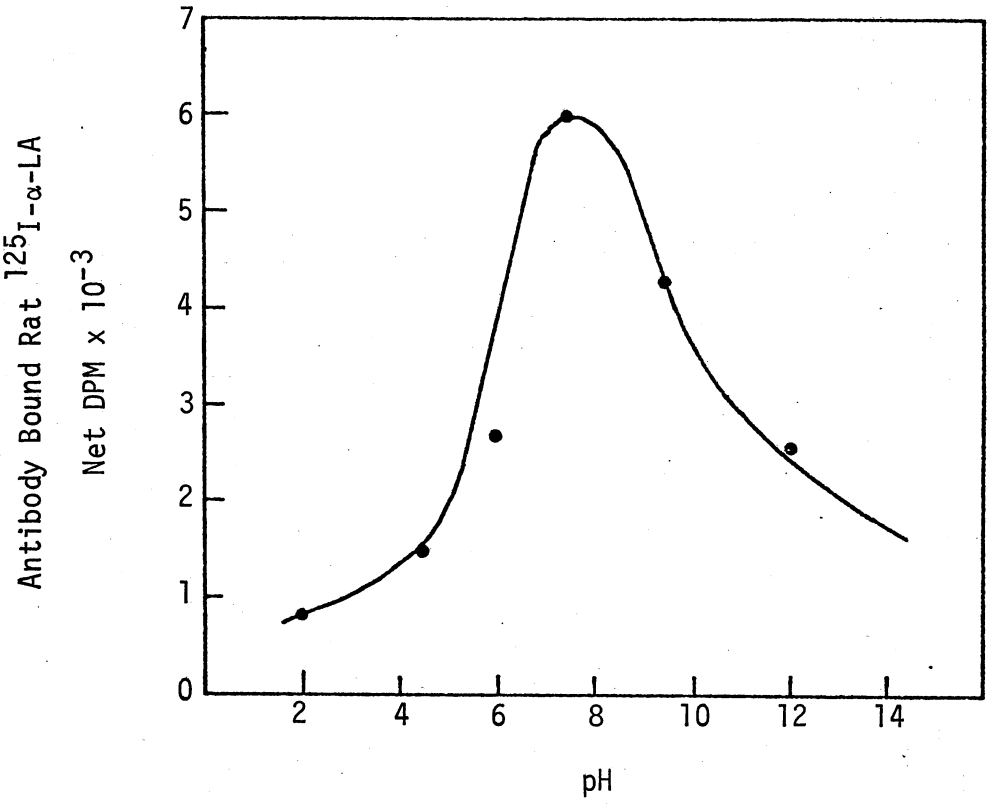

Figure 11. Effect of pH on the Rat α-Lactalbumin Radioimmunoassay

Rat ^{125}I-α-LA and antiserum were incubated at 4° C for 24 hours in 0.1 M KCl buffered at the indicated pH values. The polyethylene glycol precipitation technique was used to separate free and antibody bound rat ^{125}I-α-LA (Results).

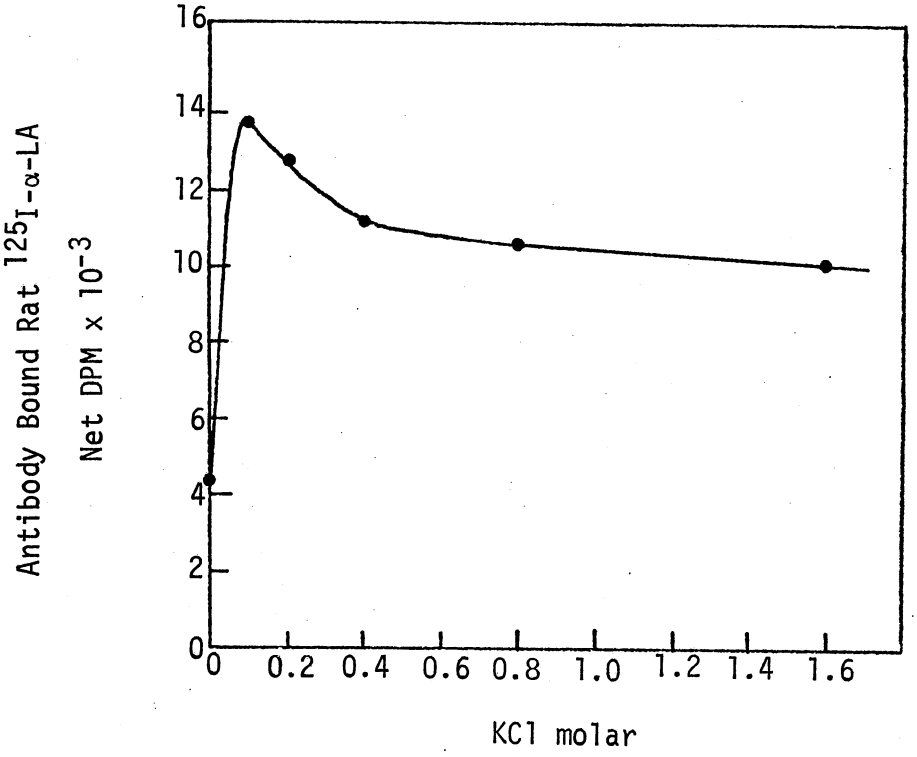

Figure 12. Effect of KCl on the Rat α-Lactalbumin Radioimmunoassay

Rat ^{125}I-α-LA and antiserum were incubated at 4° C for 24 hours in 5 mM Tris, pH 7.4 with the indicated KCl concentrations. The polyethylene glycol precipitation technique was used to separate free and antibody bound rat ^{125}I-α-LA (Results).

for each protein level were done by omitting the antirat α-LA antiserum. The samples were incubated, precipitated, washed, and counted as before. As shown in Figure 13, nearly complete inhibition of antigen-antibody binding can occur, if the protein concentration reaches high enough levels. A rather broad protein optimum occurs from about 2 mg/ml to 10 mg/ml. Protein levels below 0.5 mg/ml can also produce low net dpm, possibly due to nonspecific binding of the antigen or antibody to the glass test tubes. Combining these data and the previous data, the standard buffer solution routinely used in the RIAs is 0.1 M KCl, 20 mM Tris, pH 7.4 containing 1 mg/ml bovine serum albumin.

It was necessary to determine whether the incubation temperature and the sequence of addition of the ^{125}I-α-LA or unlabeled α-LA to the antibody would affect the level of sensitivity of the RIAs. Three sequences of addition were studied at two temperatures of 4° C and 37° C. They were (1) preincubation of the antibody with unlabeled α-LA for 12 hours followed by addition of ^{125}I-α-LA and 12 more hours of incubation, (2) preincubation of the antibody with ^{125}I-α-LA for 12 hours followed by addition of unlabeled α-LA and 12 more hours of incubation, and (3) simultaneous incubation of ^{125}I-α-LA and unlabeled α-LA with antibody for 24 hours. One hundred μl of antirat α-LA serum diluted 1:8000 in the standard buffer were mixed with either 100 μl of rat ^{125}I-α-LA containing 40,000 dpm in the standard buffer or 100 μl of standard buffer containing 0 to 40 ng of unlabeled rat α-LA. The 200 μl mixtures were incubated for 12 hours at 4° C and 37° C. Then 100 μl of rat ^{125}I-α-LA were added to the tubes containing antirat α-LA antibody and unlabeled rat α-LA. Also, 100 μl of standard buffer containing 0 to 40 ng unlabeled rat α-LA were added to the tubes containing

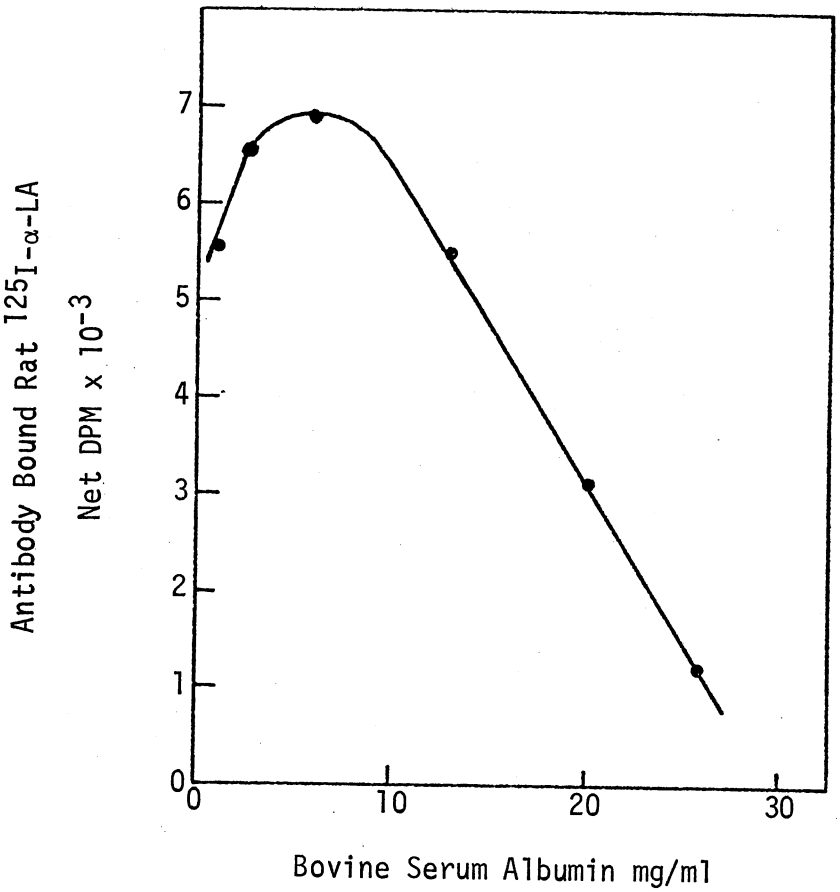

Figure 13. Effect of Bovine Serum Albumin on the Rat α-Lactalbumin Radioimmunoassay

Rat ^{125}I-α-LA and antiserum were incubated at 4° C for 24 hours in 0.1 M KCl, 20 mM Tris, pH 7.4 containing the indicated levels of bovine serum albumin. The polyethylene glycol precipitation technique was used to separate free and antibody bound rat ^{125}I-α-LA (Results).

antirat α-LA antibody and rat ^{125}I-α-LA. The 300 µl samples were then incubated for 12 hours more at the initial incubation temperature. Simultaneous incubation of 100 µl of antirat α-LA antibody with 100 µl of both rat ^{125}I-α-LA and 100 µl of standard buffer containing unlabeled rat α-LA was performed at both temperatures for 24 hours. Controls for all points were performed by omitting the antirat α-LA antibody. The solutions were precipitated, washed, and counted as before. As shown in Figure 14, the incubation temperature had little effect on the sensitivity of the assay. Incubation at 37° C caused only a slight drop in the net dpm. The significant observation is that preincubation of the antibody with the unlabeled α-LA produced the most sensitive assay. Simultaneous addition of ^{125}I-α-LA and unlabeled α-LA with the antibody extended the usable range of the standard curve. Preincubation of the antibody and ^{125}I-α-LA produced an unusable standard curve since essentially all the antibody sites were occupied by the excess ^{125}I-α-LA.

Based on these experiments the standard conditions for both RIAs are as follows. The α-LA is iodinated by the iodochloride method to a specific activity of 80 µCi/µg. The standard buffer used to dilute the ^{125}I-α-LA, the anti-α-LA serum, and the unlabeled α-LA for the standard curve is 0.1 M KCl, 20 mM Tris, pH 7.4 containing 1 mg/ml bovine serum albumin. The ^{125}I-α-LA is diluted with the standard buffer to a level of 40,000 dpm/100 µl. The anti-α-LA serum is diluted with the standard buffer to a level such that 100 µl will bind 50% of the added ^{125}I-α-LA. This dilution is 1:8000 for the antirat α-LA serum currently used, and 1:6000 for the antihuman α-LA serum. The most sensitive assay utilizes sequential addition of the antiserum and unlabeled α-LA with 12 to 24 hours of incubation at 4° C followed

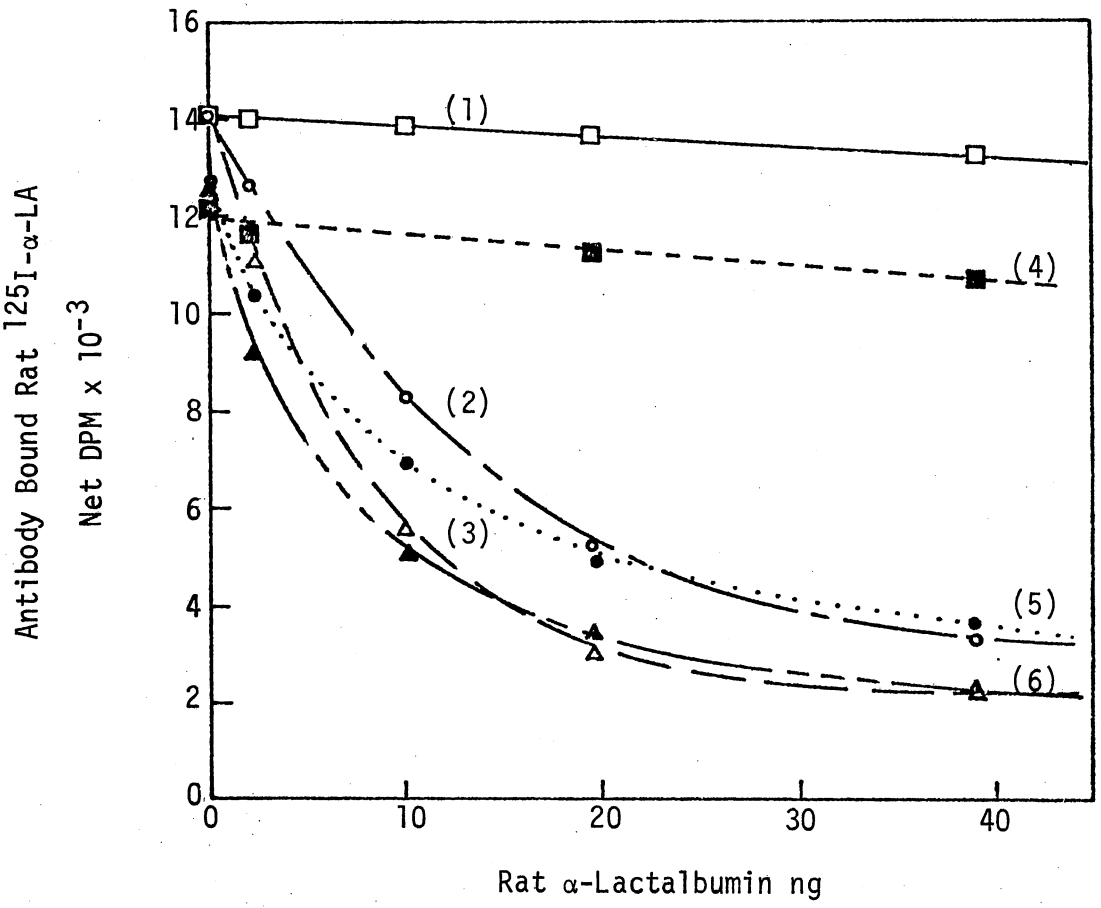

Figure 14. Effect of Temperature and Sequence of Addition of Reactants on Rat α-Lactalbumin Radioimmunoassay

Antirat α-LA serum, rat ^{125}I-α-LA, and unlabeled rat α-LA were incubated at 4° C (open symbols) and 37° C (closed symbols) using different sequences of addition. Free and antibody bound rat ^{125}I-α-LA were separated by the polyethylene glycol precipitation method (Results).

At 4° C:

(1) antibody + ^{125}I-α-LA for 12 hours, unlabeled α-LA for 24 hours

(2) antibody + unlabeled α-LA and ^{125}I-α-LA for 24 hours

(3) antibody + unlabeled α-LA for 12 hours, ^{125}I-α-LA to 24 hours

At 37° C:

(4) antibody + ^{125}I-α-LA for 12 hours, unlabeled α-LA to 24 hours

(5) antibody + unlabeled α-LA and ^{125}I-α-LA for 24 hours

(6) antibody + unlabeled α-LA for 12 hours, ^{125}I-α-LA to 24 hours

by addition of ^{125}I-α-LA with incubation at 4° C for 12 to 24 additional hours. The antiserum and ^{125}I-α-LA additions are each made in 100 μl volumes, and the unlabeled α-LA addition can be made in 100 μl or 400 μl volumes. Unknown samples must be within the limits of pH (pH 6.5 to pH 8.5), ionic strength (4 m mho to 22 m mho), and protein concentration (1 mg/ml to 10 mg/ml).

Unbound ^{125}I-α-LA is separated from antibody-bound ^{125}I-α-LA by precipitation of the antibody-^{125}I-α-LA complex with 10% polyethylene glycol. One-half ml of 0.5% bovine γ-globulin in 0.1 M KCl, 20 mM Tris, pH 7.4 is added as carrier antibody. One-half ml of 25% polyethylene glycol in water is added to the 0.3 ml RIA, and one-half ml of 32% polyethylene glycol in water is added to the 0.6 ml RIA. After vigorously mixing the sample which is then 10% in polyethylene glycol, it is placed in an ice bath for 10 minutes, then filtered through GA-8 filters, and washed twice with 2 ml of 8% polyethylene glycol. The washed filter is placed in a one inch square of aluminum foil and counted in a Packard Model 5320 gamma counter. Quenching is corrected for by applying the appropriate formula and disintegrations per minute are determined.

Controls for samples are done by omitting the antibody, and final net dpm is obtained by subtracting the sample's control dpm from the sample's experimental dpm.

Using the standard method of the RIA, typical standard curves for human and rat α-LA, shown in Figures 15 and 16, respectively, are generated.

The ability of α-LA from other species to cross-react with antibodies made to human or rat α-LA was investigated by the RIAs. Qualita-

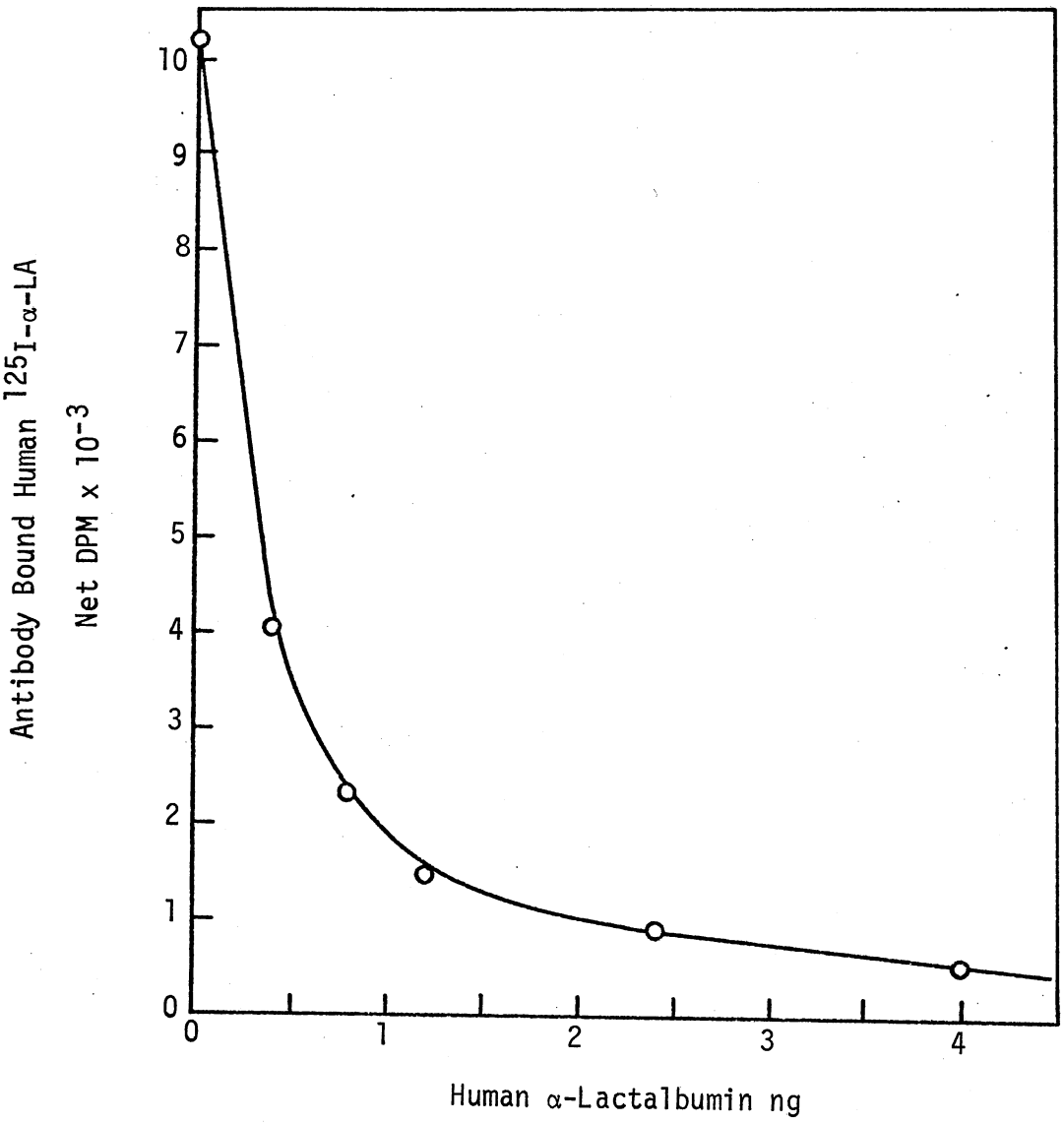

Figure 15. Standard Curve for the Human α-Lactalbumin Radioimmunoassay

Antihuman α-LA serum and unlabeled human α-LA were preincubated at 4° C for 12 hours, followed by human ^{125}I-α-LA addition and incubation to 24 hours. Free and antibody bound human α-LA were separated by polyethylene glycol precipitation method (Results).

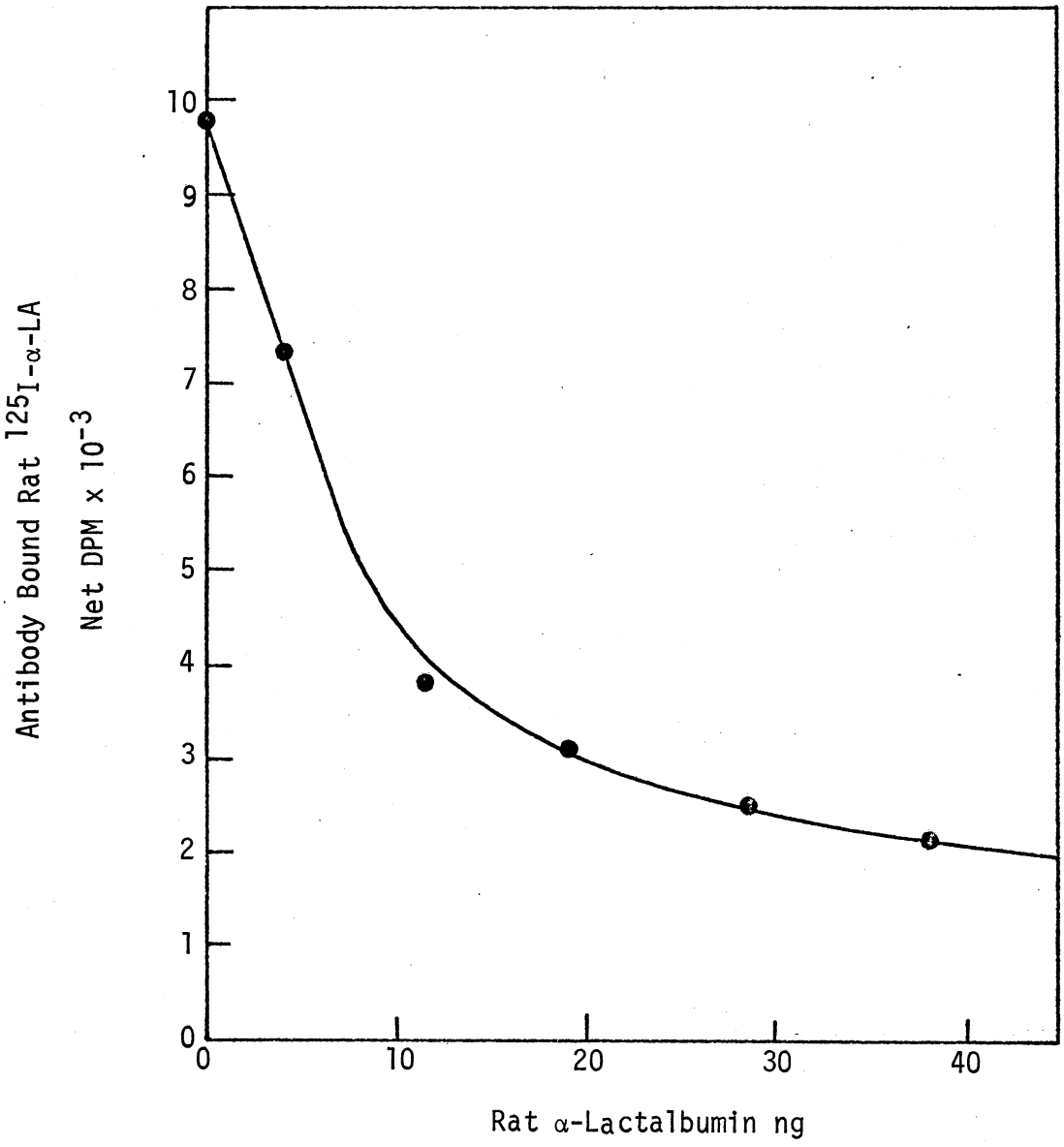

Figure 16. Standard Curve for the Rat α-Lactalbumin Radioimmunoassay

Antirat α-LA serum and unlabeled rat α-LA were preincubated at 4° C for 12 hours, followed by rat ^{125}I-α-LA addition and incubation to 24 hours. Free and antibody bound rat α-LA were separated by the polyethylene glycol precipitation method (Results).

tive results from double diffusion studies described earlier were taken a step further by determining the quantity of α-LA from the various species which produces 50% inhibition of the maximum ^{125}I-α-LA binding. Up to 100 μg of bovine, goat, guinea pig, and sheep showed no inhibition of rat ^{125}I-α-LA binding to antirat α-LA antibodies. Human α-LA indicated a slight inhibition. However, as shown in Table I, the α-LAs from other species were able to cross-react with antibodies to human α-LA, although only when present at very high levels.

TABLE I

INHIBITION$_{50}$ VALUES FOR α-LACTALBUMINS FROM DIFFERENT SPECIES DETERMINED BY THE HUMAN α-LACTALBUMIN RADIOIMMUNOASSAY

α-LA	I_{50} nanograms
Human	0.7
Rat	476×10^3
Bovine	233×10^3
Goat	25×10^3
Guinea Pig	14×10^3
Pig	28×10^3
Sheep	116×10^3

I_{50} - Level of α-LA which produces 50% inhibition of maximal trace binding

CHAPTER IV

α-LACTALBUMIN IN RAT MAMMARY TUMORS

Materials and Reagents

Rat mammary tumors were supplied by the Mason Tumor Bank as frozen whole homogenates. Trichloroacetic acid was purchased from Sigma.

Methods

The various rat mammary tumor samples which were received frozen from the Mason Tumor Bank were thawed and homogenized with a Polytron at setting six for two minutes at 25° C in 20 mM Tris, 0.1 M KCl, pH 7.4, then centrifuged at 30,000 x g for 20 minutes at 4° C. Supernatant solutions were assayed for α-LA by the standard RIA procedure.

The R3230 AC tumor was maintained by subcutaneous trocar transplantation into intact virgin female Fisher 344 rats (98). R3230 AC tumors grown for three weeks were removed and a cross-sectional segment through the center of the tumor approximately 5 mm thick was removed. Areas of the cross-sectional segment that were visually distinct were separated with scalpels in an attempt to select regions with different viability. The samples were placed in 0.1 M KCl, 20 mM Tris, pH 7.4 and homogenized at room temperature with a Polytron at setting six for two minutes. The samples were centrifuged at 30,000 x g for 20 minutes at 4° C and the supernatant solutions were assayed for α-LA by the

standard RIA procedure. Protein values for samples were determined using the modified Lowry method of Hartree (99).

It was of interest to determine if α-LA would appear in the serum of rats bearing mammary tumors. A group of virgin female Fisher rats all weighing 100 grams were injected subcutaneously by trocar implantation with explants of the R3230 AC tumor weighing 4 mg. Another group of the rats were given an intraperitoneal injection of the 13762 MAT/A ascites fluid containing 10^7 cells. After an appropriate growth period, the rat was sacrificed by decapitation and the blood collected and immediately centrifuged at 5000 x g for 20 minutes at 4° C to collect the serum. The serum was diluted four fold with 0.1 M KCl, 20 mM Tris, pH 7.4 and 400 µl of the diluted serum were assayed for α-LA by the standard RIA procedure.

To determine the half-life of α-LA in the blood, two methods were used. One hundred µl of radioactively labeled rat ^{125}I-α-LA in balanced salt solution (0.14 M NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 0.17 mM NaH_2PO_4 at pH 7.2) containing 30 ng α-LA with 250,000 dpm were injected by heart puncture into 150 gram virgin female rats anesthetized by intramuscular administration of pentabarbital (0.1 ml/150 g). Samples of blood were drawn by heart puncture at various times after ^{125}I-α-LA injection, and 200 µl were precipitated with 1 ml of 20% (w/v) trichloroacetic acid at 4° C. The samples were centrifuged at 30,000 x g for 15 minutes at 4° C and the supernatant solution and pellet were separated and both were counted in a Packard gamma counter. Intact α-LA molecules, both iodinated and non-iodinated are totally insoluble in 10% trichloroacetic acid at 4° C. The other method used 6 mg of unlabeled bovine α-LA dissolved in 200 µl of balanced salt solution which were injected by heart

puncture into 150 gram male rats anesthetized by intramuscular administration of pentabarbital (0.1 ml/150 g). One ml samples of blood were drawn by heart puncture at various times after α-LA injection and immediately centrifuged at 5000 x g for 20 minutes at 4° C. Four hundred µl of the serum were assayed for α-LA using the spectrophotometric method.

To determine the organs that are active in clearing α-LA from the blood, labeled rat ^{125}I-α-LA was injected into male rats as before. At 10 minutes, 3.5 hours, or 9 hours after injection of rat ^{125}I-α-LA, the rats were sacrificed by decapitation and drained of all possible blood. The entire organs of interest were removed, weighed, and counted in a Packard gamma counter. If it was impossible to remove the entire organ, representative areas were selected, weighed, and counted.

Results

α-Lactalbumin Levels in Rat Tumors

The Mason Tumor Bank has characterized its stock of mammary carcinomas by histological evaluation of thin sections into the broad classes indicated in Table II. Unfortunately these designations do not relate quantitatively to the biochemical status of the tumors. Utilizing the levels of the marker protein α-LA, one can evaluate whether a range of differentiation exists within the group of tumors designated adenocarcinomas.

It is interesting to note that the three tumor lines induced by DMBA showed a 56 fold difference in α-LA levels as determined by RIA. The tumor designated DMBA #14 was classified as an adenocarcinoma but had a level of α-LA only twice that of the 3M2N tumor, which is histo-

TABLE II

α-LACTALBUMIN LEVELS OF RAT MAMMARY TUMORS

Tumor	Histologic Type	ng α-LA/μg Protein
13762	Adenocarcinoma	0.096
DMBA #8	Adenocarcinoma	0.476
DMBA #14	Adenocarcinoma	0.016
R3230 AC	Adenocarcinoma	1.207
MT/W9a	Adenocarcinoma	0.413
DMBA #1(A)	Adenocarcinoma	0.894
13762 MAT/A	Ascites Carcinoma	0.016
13762 MAT/B	Ascites Carcinoma	0.009
13762 MAT/C	Ascites Carcinoma	0.024
SMT-2A	Poorly Differentiated	0.0084
3M2N	Squamous Cell Carcinoma	0.0085

logically designated as a squamous cell carcinoma. Another interesting result is seen by comparing the parent 13762 tumor, which was induced by DMBA, with its three derived ascites lines. The solid parent tumor synthesizes a rather low level of α-LA and readily metastasizes. The three ascitic lines all produce significantly lower levels of α-LA. Microscopically, the B line is characterized by a suspension of small clumps of five to ten cells. The C line, on the other hand, is a single cell suspension characterized by the lack of small clumps of cells. The C line cells are almost twice as large as the A line, vacuolated cells appear more common, and intracytoplasmic fat droplets appear to be more numerous in the C line. The A line is a mixture of the B and C line cell populations (100). The values for α-LA in the three cell line agree with their histological description.

The R3230 AC tumor has the highest α-LA level, being 1.5 times as high as the next tumor, DMBA #1a. This level of α-LA corresponds to an early lactational or late regressional normal rat mammary gland, and therefore probably represents a suitable rat model for a minimally deviated mammary adenocarcinoma.

Cross-sectional Analysis of R3230 AC Tumors

One aspect of most tumor growth is that, due to their rapid growth rate, central areas of necrosis usually develop. By selecting areas of tumors that were visually distinct, analysis of α-LA levels by RIA were made in central necrotic regions and the more viable peripheral regions. The data shown in Table III indicate that the periphery contains the highest level of α-LA and that, as one nears the focus of tumor growth, α-LA levels steadily drop. The areas of necrosis are characterized

TABLE III

α-LACTALBUMIN LEVELS IN SECTIONS OF R3230 AC TUMOR

Section	ng α-LA/μg Protein
Periphery	3.071
Second Layer	1.72
Third Layer	1.48
Center	1.02

The sections are from the periphery to the center. The periphery was about 10%, second layer 35%, third layer 35%, and center 20% of the cross section.

microscopically by local invasion of polymorphonuclear granular leukocytes, which digest the tumor cells leaving a semi-liquified debris. Realizing α-LA's resistance to proteolytic digestion, it is not unreasonable to expect some α-LA to survive the necronizing systems. Further, microscopic examination of the visually selected areas reveals an extremely sharp border between necrotic and non-necrotic areas which are impossible to cleanly separate by visual methods. This results in some overlap of tissue types between selected tissue regions.

Correlation of Serum α-Lactalbumin Levels with Tumor Growth

In 1972 Ebner and McKenzie (101) used the incorporation assay to analyze sera from male, female, and lactating rats for α-LA. They reported significant levels of α-LA in all lactating rats, with undetectable levels in male or female rats. This observation indicated that an elevated level of α-LA in the serum of a non-lactating person might be an indication of a breast lesion. To determine whether serum from tumor bearing rats would contain significantly elevated α-LA levels, sera samples from rats bearing the R3230 AC and 13762 ascites A line were collected at various stages of tumor growth. Analysis for α-LA by RIA indicated that significant serum levels of α-LA were attained as shown in Figure 17. The 13762 A line produced measurable levels of α-LA in its serum within two days after intraperitoneal injection of approximately 10^7 cells. Death for these rats occurred on days 11 and 12, but distention of the abdomen was not observable until day 4. The R3230 AC tumor took longer to produce detectable serum α-LA levels, but eventually reached higher levels. The rats had an average life span

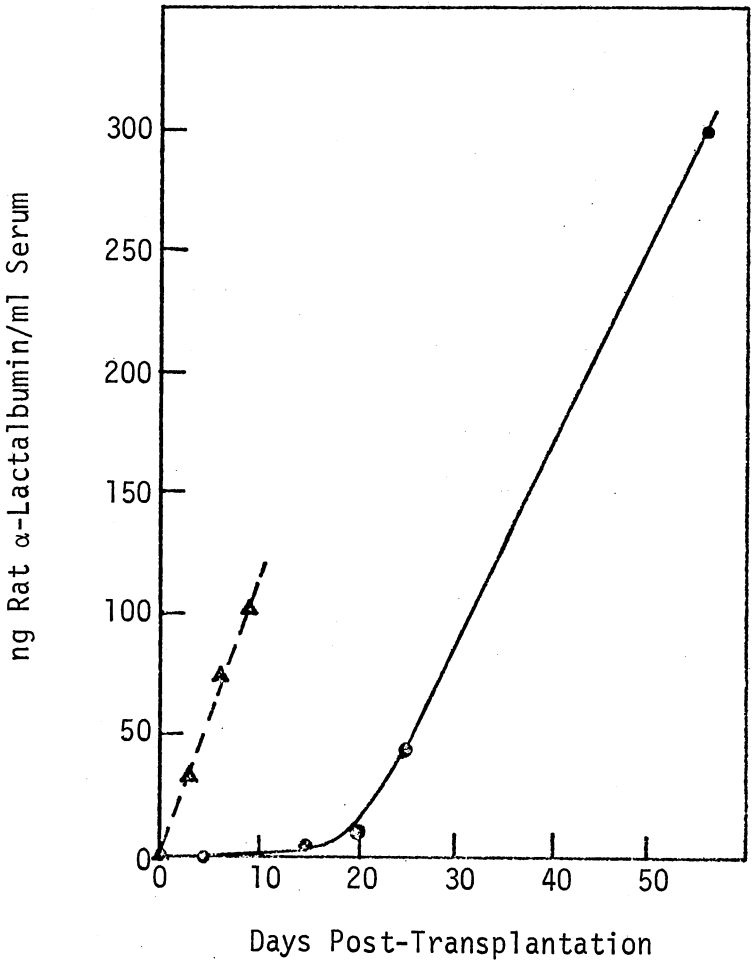

Figure 17. Levels of α-Lactalbumin in Sera of Tumor Bearing Rats

Rats were transplanted with the R3230 AC rat mammary adenocarcinoma (●——●) and the 13762 MAT/A ascities line (▲----▲). Serum samples were collected on the indicated days after injection, diluted, and assayed for α-LA by the standard RIA procedure (Methods).

following tumor transplantation of 30 days, but one survived 56 days and developed bilateral tumors which weighed a total of approximately 20 grams. Serum from a 12 day lactating rat was also assayed and the level determined was 350 ng α-LA/ml.

Clearance of α-Lactalbumin from Blood

Since α-LA was detectable in sera from tumor bearing rats, it was of interest to determine its rate of clearance from blood. The most straight-forward method was to inject rats with radioactively labeled rat α-LA and measure the decline in radioactivity in blood with time. As shown in Figure 18, the radioactivity precipitated by trichloroacetic acid, which represent intact α-LA molecules, declined in an apparent first order process with a 3.5 hour half-life. The initial trichloroacetic acid soluble radioactivity, which probably represent oxidation fragments of α-LA produced by the iodination reaction or by radiation damage, also drop at approximately the same rate. If proteolysis of α-LA were occurring in the blood, or if the organs which remove α-LA from blood were metabolizing it and secreting small fragments back into the blood, one would expect to see an increase in trichloroacetic acid soluble radioactivity. Iodinated α-LA is readily recognized by antibodies to unlabeled α-LA, indicating that the conformation of the determinants is preserved after iodination. However, to be certain that the iodination had not introduced a molecular alteration which would change the clearance rates, normal α-LA was also tested. Bovine α-LA was injected by heart puncture into a rat and sera samples drawn at various times after injection were assayed for α-LA specrophotometrically. Again the half-life was determined to be 3.5 hours, which is

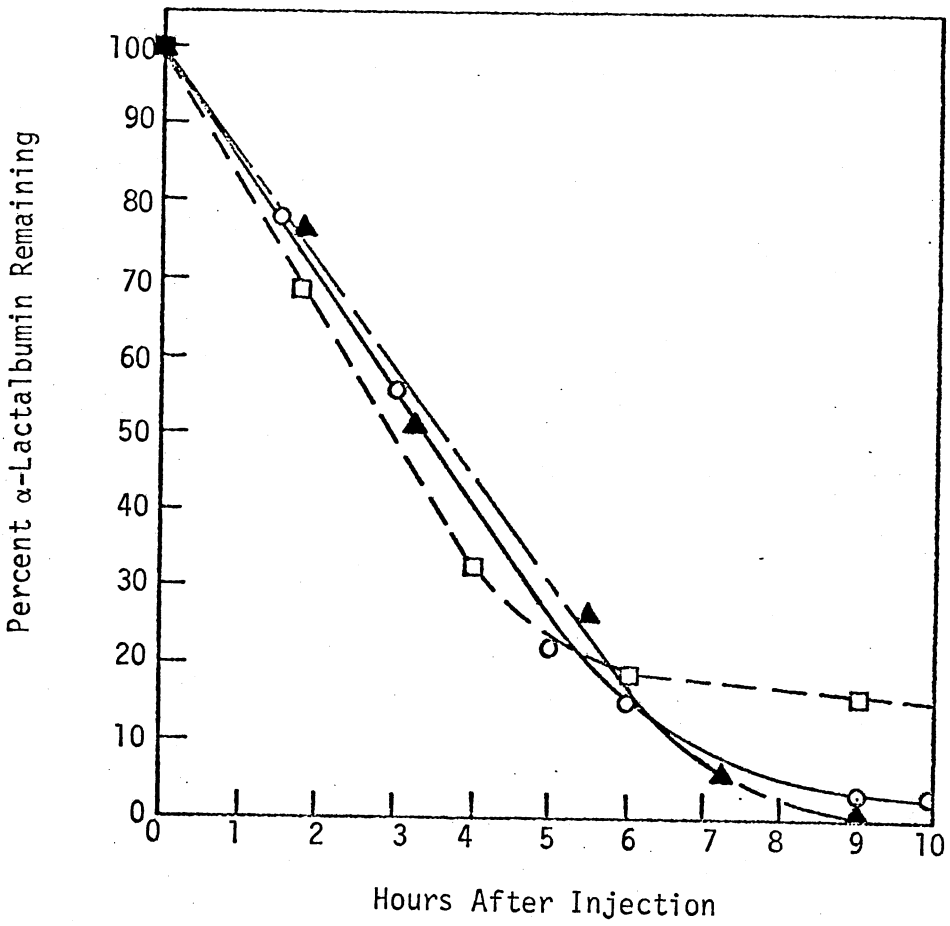

Figure 18. Clearance Rate of α-Lactalbumin from Blood

One hundred μl of rat ^{125}I-α-LA in balanced salt solution containing 240,000 dpm of trichloroacetic acid insoluble α-LA molecules and 10,000 dpm of trichloroacetic acid soluble material were injected by heart puncture into normal virgin female rats. Blood samples were drawn at the indicated times after injection. The samples were precipitated by 20% trichloroacetic acid at 4° C, centrifuged at 30,000 x g for 20 minutes and the supernatent solution (o——o) and the pellet (□- - -□) analyzed in a gamma counter. Six mg of bovine α-LA in 200 μl of balanced salt solution were also injected by heart puncture into male rats. Blood samples were drawn by heart puncture and immediately centrifuged at 5,000 x g for 20 minutes. The sera were assayed for α-LA by the spectrophotometric method at 340 nm (▲—— — ——▲) (Methods).

identical to the half-life determined for the iodinated α-LA, and the clearance seemed to be first order. The amount of radioactively labeled α-LA injected was approximately 30 ng, whereas 6 mg of nonlabeled α-LA were used for the latter experiment. Since both levels had half-lives of 3.5 hours, this is supportive evidence that the clearance is a first order process which is independent of the level of α-LA.

The organs that are responsible for the clearance of α-LA from the blood are shown in Table IV. Due to instrument failure calculation of disintegrations per minute were not possible, but based on previous experiments, a low consistent level of quench is observed in tissues. The liver has the highest total cpm followed closely by the kidneys and the lungs. Then there is a sharp drop in cpm to organs such as muscle and spleen which contain extensive capillary beds. Feces count rates were also low but positive. Collection of the urine revealed a deceiving count rate for valid α-LA. Greater than 96% of the counts in urine are not trichloroacetic acid precipitable, indicating that the trace's endogenous trichloroacetic acid soluble material, which represented about 4% of the total cpm injected, is probably filtered by the kidney. Total calculated recovery of the injected ^{125}I-α-LA from the organs surveyed was about 92%, and in no experiment did the trichloroacetic acid soluble counts appearing in the urine exceed the total trichloroacetic acid soluble counts injected. This result is congruent with the inability to detect α-LA by RIA in the urine of normal lactating rats. Sacrificing the rats at various times after injection of the labeled α-LA did not alter the relative levels of radioactivity seen in the different organs.

These data suggest that a great range of differentiation, as evalu-

TABLE IV

CLEARANCE OF SERUM α-LACTALBUMIN BY VARIOUS ORGANS

Organ	Total cpm	cpm/gm Tissue
Liver	5979	1101
Kidneys	2091	1772
Lungs	3187	3451
Spleen	517	1436
Leg Muscle*	485	346
Fat*	409	221
Heart	441	882
Brain	185	102
Cecum	392	101
Small Intestine*	241	1147
Feces	350	918
Pancreas	625	946
Ovary	198	1980
Uterus	274	782
Urine	642	802
Urine TCA Precipitant	25	31
Stomach	634	576

*Representative portion of organ.
TCA - trichloroacetic acid

ated by α-LA levels, exists in rat mammary carcinomas used as models for human breast cancer. In addition, since α-LA is found normally only in the sera of lactating rats, abnormally high serum α-LA levels, such as those found in tumor bearing animals, might be diagnostic for a breast lesion. However, the ability of many organs to clear large amounts of α-LA from the blood in a short time keeps the serum α-LA levels low, except in highly secretatory periods. Unfortunately the kidneys do not appear to clear valid α-LA to the urine.

CHAPTER V

α-LACTALBUMIN AND HUMAN BREAST DISEASES

Materials and Reagents

Fresh human tissues were obtained from the departments of Surgery and Surgical Pathology at Kansas University Medical Center. Pre-surgery sera from breast cancer patients were obtained from the files of the Detection Center for Breast Disease at Kansas University Medical Center and control sera were collected from normal male and female volunteers. Sera were also collected from women at various periods of the lactational cycle. The cultured human cell lines were supplied by the Mason Tumor Bank and were grown under recommended conditions. Cells harvested from logarithmic growth phase were termed normal growth conditions. To examine the effect of cell density on α-LA levels, cells were grown at high density (10^8 cells/cm^2) and low density (10^6 cells/cm^2) with insulin, hydrocortisone, and prolactin all present at a 5 μg/ml concentration. The solid tumors, produced by injection of human cell culture lines into the nude mouse, were supplied by Dr. B. Giovanella, the Stehlin Foundation for Cancer Research, Houston, Texas.

The P-50 ultra-filtration centrifuge cones with an approximate molecular weight exclusion limit of 50,000 were purchased from Amicon. Pepsin was purchased from Curtis-Matheson Chemical Corporation as a crude protein fraction.

Methods

The cultured human cell lines were received as a centrifuged pellet of cells which had been quickly frozen and shipped in dry-ice. The cells were thawed and resuspended in a small volume of 20 mM Tris, 0.1 M KCl, pH 7.4, then homogenized with a Polytron at setting five for two minutes at room temperature, and centrifuged at 30,000 x g for ten minutes. The supernatant solution was assayed for α-LA by the standard RIA procedure. The solid tumors produced in the nude mice by subcutaneous injection of cultured cells from human lines, as well as fresh tumors removed by surgery, were received frozen and intact. This allowed for selection of non-necrotic areas of tissue. They were also homogenized and centrifuged by the procedure described above.

Sera from patients with breast carcinomas and control sera were centrifuged in the Amicon P-50 ultra-filtration centrifuge cones at 950 x g for 60 minutes in a swinging bucket rotor number 849 with the International centrifuge, model number PR-2. These cones effectively removed all the serum proteins with molecular weights greater than 50,000, while allowing the smaller α-LA molecules to be filtered through and collected with the serum's liquid fraction. The cones were pre-treated by washing with deionized H_2O, and were reconditioned by over-night incubation in 0.1% pepsin solution followed by an extended soaking in a 1 M NaCl, 10% ethanol solution.

Urine from lactating women 1 to 3 days postpartum and urine from normal control subjects were cooled to 4° C and centrifuged at 30,000 x g for 20 minutes to remove any precipitate. The supernatant solution was assayed directly for α-LA by the standard radioimmunoassay method.

Results

Analysis of Primary Human Tumors

Although knowledge of the biochemical degree of differentiation of human mammary carcinomas would obviously be very important, no one to date has measured primary mammary tumors for α-LA or casein. Based on the data from the rat model systems, one might expect a wide range of differentiation among breast carcinomas. Frozen tissue from primary mammary carcinomas is difficult to obtain, but a reasonable number were assayed for α-LA by RIA. As shown in Table V, a rather high percentage of the breast carcinomas had detectable levels of α-LA, but these levels were low when compared to the highly secretatory R3230 AC or DMBA tumors. Histological examination of the tissues designated as breast carcinomas by pathologists revealed a wide range of cellular structures which ranged from adenoid to fatty, and gave correspondingly wide ranges of α-LA. Normal non-lactating breast tissue, which contains few epithelial cells surrounded by many fat cells, was negative for α-LA. The two cases of lymph node metastases from α-LA positive primary tumors were also positive. In addition, the metastases closely resembled the parent tumors in cellular architecture, as evaluated by hematoxylin and eosin staining. Of considerable significance was that all the benign fibrocystic, fibroadenoma, or adenoma tumors produced α-LA. The mucinous ovarian carcinoma was α-LA positive and may represent an ectopic production of α-LA, but the pancreatic tumor, lung carcinoma, and spleen myelofibrosis were negative. The case of male gynecomastia was negative. This benign disease occurs in response to excess estrogen and is the male analogue of cystic hyperplasia in the

TABLE V

α-LACTALBUMIN LEVELS IN HUMAN TUMORS

Patient	Disease / Tumor	pg α-LA/μg Protein
BO	Breast Carcinoma	1.8
BO	Normal Breast	0.0
SW	Breast Carcinoma	2.5
CO	Breast Carcinoma	22.0
CO	Metastases to Lymph Node	24.0
CN	Breast Carcinoma	0.0
VI	Breast Carcinoma	0.0
FO	Breast Carcinoma	0.0
PO	Breast Carcinoma	3.2
PO	Metastases to Lymph Node	19.1
WA	Breast Carcinoma	0.4
HO	Breast Carcinoma	1.6
GA	Breast Carcinoma	4.7
DY	Fibrocystic Disease	2.6
DV	Fibrocystic Disease	116.0
LA	Benign Adenoma	25.0
HE	Fibrocystic, Fibroadenoma	12.2
HA	Fibrocystic Disease	36.1
AR	Fibrocystic Disease	1.3
MI	Fibroadenoma	6.5
BU	Spleen Myelofibrosis	0.0
HA	Lung Carcinoma	0.0
SU	Male Gynecomastia	0.0
JA	Pancreatic Carcinoma	0.0
RO	Ovary Carcinoma	7.6

female.

Correlation of α-LA levels to classic examples of breast diseases would be an obvious aid in clinical differential diagnosis of breast masses which determines the correct operative treatment.

Analysis of Human Cell Culture Lines

In addition to maintaining a depository of rat model tumors, Mason Tumor Bank has attempted to place purported human mammary cells from several sources into stable cultured lines. The general procedure is to obtain cells by pleural effusion from patients with different types of breast carcinomas, based on a theory that the cells comprising the pleural fluid will be derived from the primary tumor. This technique offers the advantage of providing cells that are already dispersed into single cells, rather than having to mechanically or enzymatically disperse a solid tumor into isolated cells, which one could then attempt to place into cell culture. Some objections to the pleural effusion technique have been raised. One point in question is, can one be reasonably sure that the majority of cells comprising a pleural effusion are valid mammary carcinoma cells. Further, even if the cells are spawned by the primary tumor, do they still retain any biochemical similarity to the original tumor, or have they undergone significant alterations of gene expression which are inherent prerequisites demanded by the metastasizing process. These pertinent questions have not been answered. The presence of α-LA in the cells could help to establish their parentage and their level of differentiation. Indeed, there have been some indications that human lines in culture which were thought to be mammary cells are actually Hela cells (4).

In an attempt to establish the cell parentage and degree of differentiation of the cell lines carried by the Mason Tumor Bank, samples were analyzed for α-LA by the RIA. As shown in Table VI, three lines were obtained by pleural effusions of different types of mammary carcinomas, the HBL-100 line was obtained from cells present in breast milk, and a line was obtained from normal amnion fluid. None of the lines contained levels of α-LA significantly above the limit of detection, i.e. above 0.3 pg α-LA/μg protein, when harvested in logarithmic growth phase without hormones. Since normal breast tissue is not actively dividing while synthesizing milk proteins, it was of interest to determine whether slower growth rates and hormones could stimulate the cells to form α-LA. Cells were grown in high and low density in the presence of hormones insulin, hydrocortisone, and prolactin, and then assayed for α-LA by RIA. These modifications of growth conditions were not successful in raising α-LA levels above background. Since neither the original parent tumors nor the original pleural effusions were analyzed for α-LA, one cannot be sure whether the cells were ever sufficiently differentiated to synthesize α-LA, or whether the cells lost the ability due to the non-physiological conditions inherent with *in vitro* culturing.

Another ingenious method for slowing the rate of cell replication, for replacing cell-cell contact, and for providing nutrients found in blood to human tumor cells, is to employ the nude, or athymic, mouse as a growth vehicle. Since this mouse strain has essentially no cell mediated immunological response, foreign tissues from antigenically different species are not usually rejected. This allows one to place human cells back into an *in vivo* system with very nearly normal physio-

TABLE VI

α-LACTALBUMIN LEVELS OF HUMAN CELL CULTURE LINES

Cell Line	Cell Origin	pg α-LA/μg Protein		
		Log Growth	High Density	Low Density
HBL-100	Normal Breast Milk	<0.3	<0.3	<0.3
MB-134 VII	Pleural Effusion of Infiltrating Ductal Carcinoma	<0.3	<0.3	<0.3
MB-157	Pleural Effusion of Metastasizing Medullary Carcinoma	0.6	<0.3	<0.3
MB-231	Pleural Effusion of Poorly Differentiated Papillary Carcinoma	0.3	0.3	<0.3
FL-amnion	Normal Epithelial Cells from Amnion Tissue	0.5	---	---

logical conditions which are unattainable in cell culture. Following subcutaneous injection of the tumor cells, solid tumors were produced which were analyzed for α-LA by RIA. Comparison of α-LA levels from the cells grown in culture conditions with the α-LA levels in solid tumors would indicate whether factors critical for expression of differentiated functions are missing under in vitro conditions of cell culture. Table VII indicates that the BT-20 cell line does not have measurable α-LA levels in cell culture, nor do the solid tumors produced by the cell lines designated CO-1, CL-1, and ST-1. Analysis of the solid tumors produced by the BT-20, KE-1, and GI-1 cell lines have given variable results. One set of tumors produced significant levels of α-LA, but the second set of tumors produced undetectable levels. Histological techniques to be discussed later supported the negative RIA results.

Analysis of Sera from Breast Cancer Patients

Analysis of the primary tumors and their metastases indicated a significant percentage synthesize detectable levels of α-LA. Analysis of presurgical sera could indicate what proportion of breast carcinomas synthesize α-LA at a level that is sufficient to produce a significant increase in serum α-LA concentration. As pointed out previously, RIAs can give false positive values if non-specific protein levels reach high enough levels, such as found in non-diluted serum. To avoid this problem, sera were filtered through Amicon ultra-filtration centrifuge cones which allowed small molecular weight components such as α-LA to pass through while retaining compounds with molecular weights greater than 50,000 (102). The protein concentration following ultra-filtra-

TABLE VII

α-LACTALBUMIN LEVELS OF HUMAN CELL LINES GROWN IN NUDE MICE

Cell Line	Cell Origin	pg α-LA/μg Protein Sample 1	Sample 2
CL-1	Primary Culture of Carcinoma	<0.3	<0.3
CO-1	Eighth Passage of Primary Culture	<0.3	<0.3
KE-1	Pleural Effusion of Breast Carcinoma	600	---
BT-20 Cells	Fifteenth Year of Culturing	<0.3	<0.3
BT-20 Solid	Fifteenth Year of Culturing	530	<0.3
G1-1	Pleural Effusion of Breast Carcinoma	120	---
ST-1	Pleural Effusion of Breast Carcinoma	---	<0.3

tion is consistently within a range of 1 mg/ml, which is ideal for the RIA. Recovery of α-LA was rigorously checked by adding known amounts of bovine α-LA to serum and then monitoring its recovery in the filtrate. The cones exhibited a consistent recovery of 83 ± 5%, and were periodically rechecked for recovery level and cleaned after each usage by a pepsin digest with an alcohol and salt wash. Analysis of 38 presurgical breast carcinoma sera indicated that ten had α-LA levels significantly elevated above the control sera as shown in Table VIII. Male control sera were all negative. By examining sera taken from normal women at different points during the menstrual cycle who were or were not using birth control pills, it was determined that there is no normal rise and fall of α-LA in their serum which can be attributed to normal physiological processes. Analysis of postsurgical sera from the patients who had elevated presurgical serum α-LA levels indicated no detectable level of α-LA, indicating removal of the tumor removed the source of α-LA production. The level of detection of α-LA in serum is 0.15 ng α-LA/ml and values below this level are reported as 0.0.

Analysis of Urine from Lactating Women

The kidneys effectively filter small molecules across the glomerular basement membrane. Myosin, which has a molecular weight of 17,500, has a glomerular filtration ratio of 0.97 indicating it is readily filtered into the glomerulus (103). Since α-LA has a smaller molecular weight, one would suspect it would also be filtered from the blood. Normal urine has approximately 20 μg protein/ml and a conductivity of 12 m mho, both of which are within the optimum ranges for the RIA. If α-LA is filtered by the kidneys, it probably would be in higher concen-

TABLE VIII

α-LACTALBUMIN LEVELS OF SERA FROM BREAST CARCINOMA PATIENTS

Patient	ng α-LA/ml Presurgery	Postsurgery
M-1, W-1, W-3, W-4, W-5,	0.0	---
W-7, W-8, W-9, W-10, W-11	0.0	---
W-12, W-13, W-14, W-15,	0.0	---
U-1, U-2, V-1, V-2, LO,	0.0	---
SL, SV, SR, SW, SM, SP	0.0	---
WO	2.81	0.0
VA	2.50	0.0
UH	2.50	0.0
YO	3.75	0.0
SH	4.06	0.0
SE	2.50	0.0
SC	5.31	0.0
U-1	1.25	0.0
W-6	59.8	---
W-2	17.3	---

Controls	ng α-LA/ml
1-M, 2-M, 1-F, 2-F	0.0

1-M, 2-M - normal male sera
1-F, 2-F - normal female sera

tration in the urine than it is in serum, and urine samples would not have to be deproteinized before they could be analyzed by RIA. Samples from six lactating women who were one to three days postpartum were analyzed by RIA along with urine from four normal males and three normal females. As shown in Table IX, no α-LA was detectable in the controls or lactating urines. To ensure that some compound present in urine was not prohibiting detection of α-LA, a known amount of α-LA was added to a lactating urine, and no inhibition of its detection was noted. These data strongly indicate that, although α-LA is theoretically small enough to be filtered by the glomerulus, α-LA is not collected in urine.

TABLE IX

α-LACTALBUMIN LEVELS OF URINE FROM LACTATING WOMEN

Subject	Condition	ng α-LA/ml
RO	Lactating	0.0
MU	Lactating	0.0
KE	Lactating	0.0
CL	Lactating	0.0
BI	Lactating	0.0
HA	Lactating	0.0
LB	Male Control	0.0
GS	Male Control	0.0
CG	Male Control	0.0
IN	Male Control	0.0
SS	Female Control	0.0
CC	Female Control	0.0
RS	Female Control	0.0
HA	Lactating + α-LA (4 ng)	4.0
HA	Lactating + α-LA (8 ng)	8.0

CHAPTER VI

ANALYSIS OF RAT AND HUMAN TUMORS FOR α-LACTALBUMIN BY HISTOCHEMICAL METHODS

Immunofluorescence System

Materials and Reagents

Fluorescein labeled goat antirabbit γ-globulin was purchased from Pentex. Rat serum albumin was purchased from Sigma. Slides were viewed on a Zeiss fluorescent microscope Model RA equipped with FL barrier filter inserts, Neofluor objectives, and HBO-200 illuminator. Photographs were taken with a PM-10 Olympus 35 mm camera using Kodak EHB-135 film. A Cryo-Cut cyrostat manufactured by American Optical Company was used to cut frozen sections. Balanced salt solution was 0.14 M NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 0.17 mM NaH_2PO_4 at pH 7.2.

Methods

Detection of antigens by the indirect or Coons (76) method has been exploited for numerous systems. In general terms the basic procedure is to apply a solution which contains antibodies directed against the desired antigen on a thin section of the tissue to be tested. After an appropriate incubation period, the tissue section is washed until the unbound antibodies are removed, and a second fluorescein labeled antibody solution which is directed against the first antibody molecules is

applied. After further incubation and washing, the sections are mounted in glycerol and viewed with an ultraviolet fluorescent microscope. Intensity of fluorescence is judged to 0.5 units on a unitless scale of increasing fluorescence from 0 to 8 by double blind evaluation of two investigators. Values were reported as the average of the two investigator's values.

The procedure used for the immunofluorescence system was as follows. Specimens of frozen tumor tissue were cut on a cryostat at -20° C producing 4 to 6 micron thick sections. Due to its fatty nature, it was necessary to cut lactating mammary gland tissue sections at 10 to 14 micron thickness. The sections were placed on glass slides and air-dried for 30 minutes at room temperature. The slides were placed in 10% neutral buffered formalin (10% formaldehyde (v/v) in 50 mM phosphate, pH 7.0) for either 5 minutes or 18 hours. After fixation, they were washed at room temperature in balanced salt solution for 1 hour. The anti-α-LA serum and the normal rabbit serum were diluted 10 fold with balanced salt solution, male rat serum or male human serum. The fluorescein-labeled goat antirabbit γ-globulin was diluted 50 fold with balanced salt solution.

Four slides were necessary for each experimental condition and were designated immune serum, normal rabbit serum, goat antirabbit γ-globulin, and autofluorescence. The immune serum slide and normal rabbit serum slide, which serves as a control, received their respective serum followed by the fluorescein-labeled goat antirabbit γ-globulin serum. The control slide designated goat antirabbit γ-globulin received no rabbit serum treatment but received only the goat antirabbit γ-globulin serum. The autofluorescence control slide had no serum treatment.

Performing all incubations and washings at room temperature, the immune serum, containing anti-α-LA antibodies, and normal rabbit serum were applied to their slides for 30 minutes, and then washed for 30 minutes in balanced salt solution. The fluorescein labeled goat anti-rabbit γ-globulin serum was applied to the immune serum slide, to the normal rabbit serum control slide, and to the goat antirabbit γ-globulin control slide. The three slides were incubated for 30 minutes and washed for 30 minutes in balanced salt solution. All four slides were then mounted in glycerin and evaluated by the double blind procedure.

During the development of this final optimized procedure for demonstration of α-LA by immunofluorescence, several important observations were made. Treatment of mammary tissue by the different fixing reagents acetone, ether/alcohol, acetic acid, and formalin indicated that 10% neutral buffered formalin produced the best tissue preservation with the least introduction of autofluorescence. Because frozen mammary tissue is extremely difficult to section thinly, attempts were made to use the paraffin embedding process. Sections 5 microns thick can easily be cut from paraffin blocks, and tissue preservation is excellent. However, mammary tissue develops severe autofluorescence during the dehydration process, and thereby renders paraffin sections totally unusable for fluorescence studies. It was necessary, therefore, to utilize only frozen tissue samples.

Because both the antirat α-LA and the antihuman α-LA sera have high titers, a 10 fold dilution of the antisera produced fluorescence as intense as nondiluted serum, but a 20 fold dilution was less intense. Similarly, a 50 fold dilution of the fluorescein labeled goat anti-rabbit γ-globulin was determined to be the optimal concentration.

Incubation of the sera with tissue sections at 37° C or 4° C produced less net fluorescence than at 25° C. Incubation of the test sera with the tissues produced no additional net fluorescence after 30 minutes. Similarly, 30 minute washes of the tissues were sufficient to remove nonspecifically bound γ-globulin.

As will be discussed later, it has been theorized that mammary secretory epithelial cells possess specific γ-globulin receptors on their plasma membranes. Since the vehicle used to recognize the desired antigen is a γ-globulin molecule, this adds another dimension to immunofluorescence on frozen sections of mammary gland. If the γ-globulin receptors of the test tissue recognize the γ-globulins from the species used to generate either the first or second antibody, then binding and finally fluorescence will occur whether or not the particular antigen is present. This problem can be resolved in two ways. One can attempt to block the γ-globulin receptors with a γ-globulin to which the second fluorescein labeled antibody does not cross-react. Alternatively, one can try to inactivate the γ-globulin receptor with denaturents which do not destroy the determinants of the antigen in question. Both methods were successfully employed.

For the following experiments, two objectives were in mind: first, to detect α-LA in tissues, and second, to demonstrate the ability to block the γ-globulin receptors or destroy their activity. Four rat tissues were investigated; normal mammary gland from a 12 day lactating Fisher rat, R3230 AC tumor, solid 13762 tumor produced by subcutaneous injection of 13762 ascites cells of the MAT/A line, and 13762 ascites cells of the MAT/A line. Two human mammary tissues were investigated; a fibrocystic disease and an adenoma. All were sectioned and fixed as

described before.

Results

To determine the spectrum of cross-reactivity of the fluorescein labeled goat antirabbit γ-globulin, double diffusion tests were carried out at 25° C in 1% agar in 0.1 M KCl, 20 mM Tris, pH 7.4. The antigens tested were undiluted male rabbit serum, male rat serum, male human serum, and bovine γ-globulins at 10 mg/ml, bovine serum albumin at 30 mg/ml, and rat serum albumin at 30 mg/ml, all in balanced salt solution. Only male rabbit serum produced any precipitate band, indicating that the fluorescein labeled goat antirabbit γ-globulin antibodies would recognize only rabbit γ-globulins. This would allow an attempt to block the γ-globulin receptors on rat and human mammary tissues with several γ-globulins.

By performing simultaneous experiments on tissue sections which had been fixed in formalin for 5 minutes or 18 hours and by using antibody solutions which did or did not contain blocking γ-globulins, it was possible to demonstrate (1) the presence of α-LA; (2) the susceptibility of the γ-globulin receptors to chemical modification; and (3) the ability to block the γ-globulin receptors with γ-globulins to which the fluorescein labeled goat antirabbit γ-globulin antibodies did not cross-react. As shown in Table X, the sections of lactating rat mammary gland that were fixed for 5 minutes had a net difference in fluorescence of 0.7 for sections treated with immune serum and normal rabbit serum when rat serum was not present to block the γ-globulin receptors with rat γ-globulins. By incubating the sections fixed for 5 minutes in formalin with the test sera that contained male rat serum, the net fluorescence was

TABLE X

EVALUATION OF α-LACTALBUMIN IN NORMAL AND RAT MAMMARY CANCERS BY IMMUNOFLUORESCENCE

Tissue	Test Serum	5 min NBF Antiserum Diluted in Balanced Salt	5 min NBF Antiserum Diluted in Male Rat Serum	18 h NBF Antiserum Diluted in Balanced Salt	18 h NBF Antiserum Diluted in Male Rat Serum
Lactating Rat Mammary Gland	IS	4.2	4.5	5.0	4.5
	NRS	3.5	1.2	2.2	1.2
	GARγg	0.0	0.0	0.0	0.0
	Net	0.7	3.3	2.7	3.3
R3230 AC	IS	2.5	2.5	3.5	2.5
	NRS	2.0	0.2	1.5	0.0
	GARγg	0.0	0.0	0.0	0.0
	Net	0.5	2.3	2.0	2.5
13762 MAT/A Solid	IS	3.2	1.5	3.0	2.5
	NRS	2.5	0.0	1.0	0.2
	GARγg	1.0	0.0	0.5	0.0
	Net	0.7	1.5	2.0	2.3
13762 MAT/A Ascities	IS	4.0	3.0	3.0	2.0
	NRS	2.0	0.0	1.0	0.0
	GARγg	0.0	0.0	0.0	0.0
	Net	2.0	3.0	2.0	2.0

NBF - neutral buffered formalin
GARγg - goat antirabbit γ-globulin

IS - Immune Serum
NRS - Normal Rabbit Serum

increased to 3.5. The net increase was caused by the dramatic drop in fluorescence of the normal rabbit serum control slide. This indicated indirectly that the excess rat γ-globulins were effectively occupying the majority of the tissue's specific γ-globulin receptors. Since the goat antirabbit γ-globulin antibodies do not cross-react with rat γ-globulins, the nonspecific background fluorescence represented by the normal rabbit serum slide was drastically reduced. The goat anti-rabbit γ-globulin slides were all 0.0, indicating that the γ-globulin receptors on rat mammary tissue do not recognize goat γ-globulins. Further experiments with other blocking molecules indicated that bovine γ-globulins, bovine serum albumin, human γ-globulins, and rat serum albumin could not decrease the fluorescence of the normal rabbit serum control slide. This indirect evidence is complementary to the direct evidence data presented in Chapter VII which indicates that the γ-globulin receptors of rat mammary epithelial cells are specific and do not recognize human or bovine γ-globulins.

The sections incubated 18 hours in formalin showed much less evidence of active γ-globulin receptors. The section treated with normal rabbit serum which did not contain blocking rat serum had a fluorescence value of 2.2, and when excess rat serum was present, the value moderately dropped to 1.2.

The net fluorescence value indicates the level of α-LA, and identical values are obtained for both the 5 minute and 18 hour formalin treated sections if blocking rat serum is used. Omitting the rat γ-globulins produced a net increase from 0.7 to 2.7 caused by the extended formalin fixation.

As shown in the photographs in Figure 19, the immune serum produced

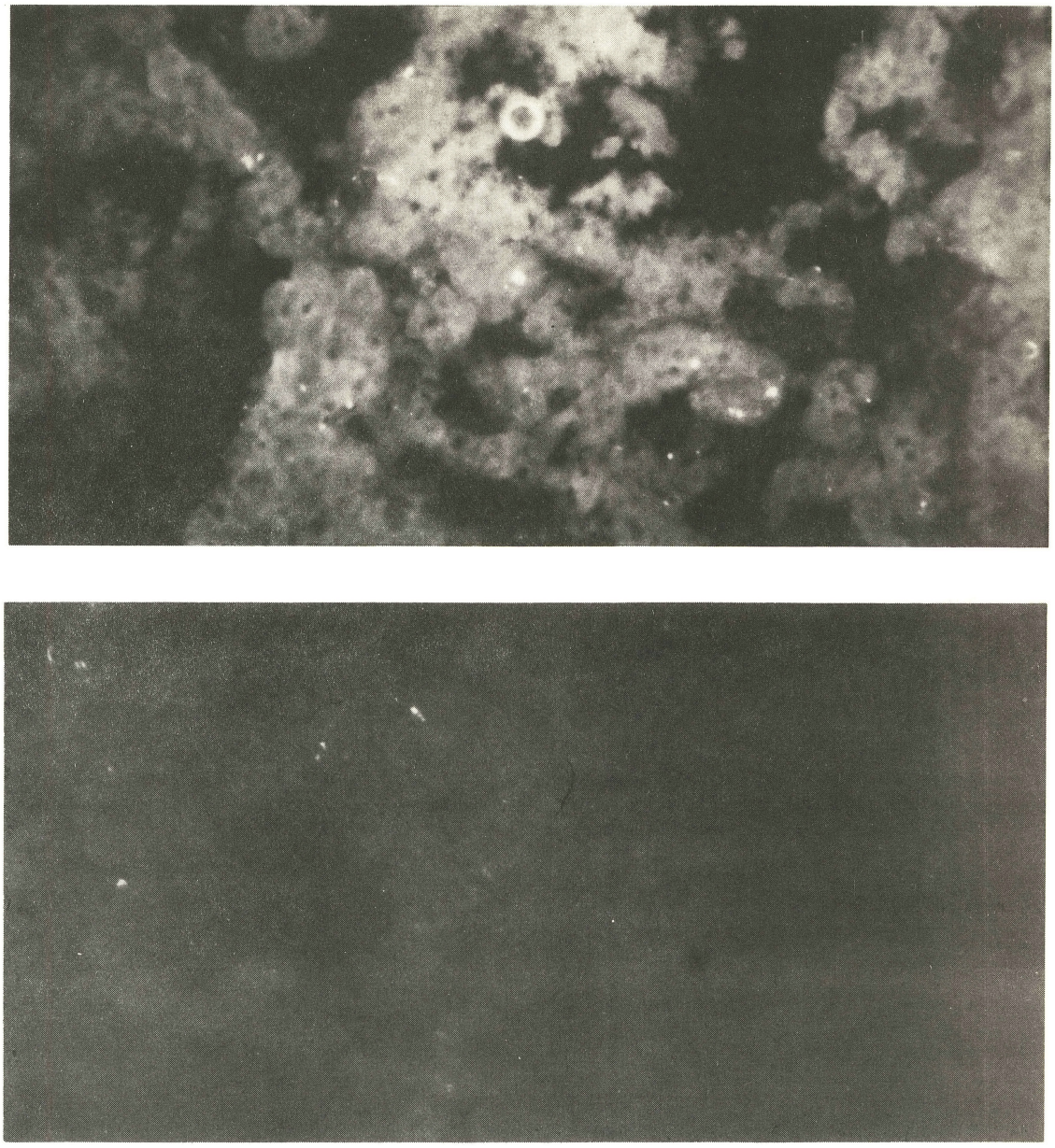

Figure 19. Photomicrographs of Lactating Rat Mammary Gland Using the Immunofluorescence Method for Detection of Rat α-Lactalbumin

Sections of lactating rat mammary gland were fixed in neutral buffered formalin for 18 hours. The top panel is tissue treated with rabbit antirat α-LA serum diluted with male rat serum followed by fluorescein labeled goat antirabbit γ-globulins. The bottom panel is tissue treated with normal rabbit serum diluted with male rat serum followed by fluorescein labeled goat antirabbit γ-globulins (Methods).

fluorescence that was located entirely in the cytoplasm. The nuclei of the epithelial cells forming the ascini appeared as black holes. The supporting stromal elements were also negative. Secretory products present in some lumen which were not washed out also fluoresced intensely. Control tissues such as rat muscle gave zero fluorescence for all three test sera.

Similar results were obtained for the other three rat tissues. All had demonstrable levels of α-LA which were in qualitative agreement with the levels determined by RIA.

Two additional experiments were performed to conclusively demonstrate the specificity of the immunofluorescence/horseradish peroxidase systems. Pure rat α-LA was added to 10 fold diluted rabbit antirat α-LA serum (4 mg pure rat α-LA/ml diluted antirat α-LA serum), then this serum was applied to sections of lactating rat mammary gland that had been fixed in formalin for 18 hours. No increase in fluorescence was observed for the α-LA treated immune serum slide above the control normal rabbit serum slide, although the untreated immune serum slide produced a net fluorescence of 3.0. This result demonstrated that excess pure rat α-LA would block all the specific fluorescence of the immune serum.

None of the supernatant solutions from homogenized normal rat tissues produced any displacement of counts in the rat α-LA RIA, indicating that there is no general species cross-reactivity between rabbit antibodies and soluble rat proteins. To eliminate the possibility that there might also have been tissue/species cross-reactivity, 1 ml samples of rabbit antirat α-LA serum and normal rabbit serum were incubated for 30 minutes at room temperature with 100 mg of normal rat liver acetone powder (104), then centrifuged for 20 minutes at 30,000 x g at 4° C.

The supernatant solutions were compared with untreated immune serum and normal rabbit serum samples on sections of lactating rat mammary gland fixed in formalin for 18 hours. Identical fluorescence values were obtained for both the liver acetone powder treated and untreated immune serum (3.5) and normal rabbit serum slides (0.5) producing a net fluorescence value of 3.0 for both the acetone powder treated slides and the untreated slides. This indicated that both the RIAs and the histochemical systems were specific for detection of α-LA.

Human tissues were also examined in the same manner for α-LA and γ-globulin receptors. Two benign tumors were obtained and one was diagnosed as fibrocystic disease and the other as an adenoma. Six micron sections were cut from the frozen specimens. These were fixed in formalin for 5 minutes or 18 hours and washed in balanced salt until used. The three human test sera were diluted in the same manner as the rat test sera except male human sera was substituted for the male rat sera as the blocking γ-globulins. As shown in Table XI, α-LA was demonstrable in both tissues, but at rather low levels. A decrease in fluorescence was seen with both chemical inactivation by formalin and with blocking of the receptor with human γ-globulins. Because the levels of α-LA seemed to be lower in human tumors than in the rat model tumors, and because fresh frozen human tissue is difficult to obtain, a more sensitive system of detecting α-LA in situ that could be applied to paraffin sections was developed.

Horseradish Peroxidase System

Materials and Reagents

Nonlabeled goat antirabbit γ-globulin was purchased from Pentex.

TABLE XI

EVALUATION OF α-LACTALBUMIN IN HUMAN CANCERS BY IMMUNOFLUORESCENCE

Disease	Test Serum	5 min NBF Antiserum Diluted in BSS	5 min NBF Antiserum Diluted in Male Human Serum	18 h NBF Antiserum Diluted in BSS	18 h NBF Antiserum Diluted in Male Human Serum
	IS	2.5	2.0	1.7	1.5
Patient DA	NRS	1.5	1.0	0.5	0.2
Fibrocystic Disease	GARγg	0.0	0.0	0.0	0.0
	Net	1.0	1.0	1.2	1.3
	IS	---	---	4.0	3.5
Patient LA	NRS	---	---	2.5	2.0
Adenoma	GARγg	---	---	0.0	0.0
	Net	---	---	1.5	1.5

IS - immune serum
NRS - normal rabbit serum
GARγg - goat antirabbit γ-globulin
NBF - neutral buffered formalin
BSS - balanced salt solution

Horseradish peroxidase type II, 3,3'-diaminobenzidine tetrahydrochloride, periodic acid, and sodium borohydride were purchased from Sigma. The 1-fluor-2,4-dinitrobenzene was purchased from Aldrich. Ethylene glycol and hydrogen peroxide were purchased from Matheson. The A 0.5 M chromatography gel was purchased from Bio-Rad. Formation of the diaminobenzidine precipitate was evaluated with an Olympus microscope Model EHT, and photographs taken using the PM-10 photographic unit. Tissue specimens were automatically processed through paraffin embedding by the Autotechnicon instrument.

Methods

The traditional method of coupling horseradish peroxidase to antibodies employed a bivalent coupling reagent, such as glutaraldehyde, to covalently attach the two proteins. Horseradish peroxidase and antibody were mixed with glutaraldehyde for several hours and the randomly coupled products were separated on molecular weight columns. This technique was subject to the major drawbacks of self-coupling of each protein to itself, and loss of biological activity by chemical modification of either or both proteins.

The new procedure which was employed circumvented these problems by allowing only directed coupling of horseradish peroxidase to antibody molecules. This resulted in a very high correct coupling efficiency, while retaining full biological activity of both proteins. This procedure, developed by Nakane and Kawoai (105), takes advantage of the glycoprotein nature of horseradish peroxidase which has a carbohydrate content of 18%. In general terms, the first step blocks the free amino groups on horseradish peroxidase by reaction with fluorodi-

nitrobenzene. Then the carbohydrate moities are oxidized with sodium periodate producing aldehyde groups which can be reacted unidirectionally with the free amino groups on the antibody molecules. The resulting Schiff's base is stabilized further by reduction with sodium borohydride, and the active coupled product is purified by column chromatography. Specifically, 5 mg of horseradish peroxidase type II were dissolved in 1 ml of 0.3 M $NaHCO_3$, pH 8.1, and 0.1 ml of 1% (v/v) fluorodinitrobenzene in absolute alcohol was added at room temperature with gentle stirring by a paper clip stirring bar. After reacting for 1 hour, 500 µl of 0.12 M $NaIO_4$ in H_2O were added and the mixture stirred for another hour at room temperature. To the resulting yellow-green solution, 500 µl of 0.32 M ethylene glycol in H_2O were added and stirred at room temperature for another hour. The solution was then dialyzed three times against a liter of 10 mM $NaHCO_3$, pH 9.5, at 4° C. The blocked and oxidized horseradish peroxidase was reacted with 5 mg of lyophilyzed goat anti-rabbit γ-globulin for 3 hours at room temperature, and the resulting Schiff's base was stabilized by reduction with 5 mg of $NaBH_4$ for 3 hours at 4° C. The solution was subsequently dialyzed against balanced salt solution overnight at 4° C, centrifuged at 30,000 x g for 10 minutes, and the supernatant solution was applied to the A 0.5 M molecular weight filtration column equilibrated with 0.1 M KCl, 50 mM phosphate, pH 7.4. One ml fractions were collected at a 4 s/drop rate and the protein elution profile determined by absorbancy at 280 nm. Horseradish peroxidase was detected by absorbancy at 403 nm. Samples were stored at -20° C with no loss of either biological activity.

The system for detecting α-LA with the horseradish peroxidase coupled goat antirabbit γ-globulin is based on the same principles as

the fluorescein labeled goat antirabbit γ-globulin system. Briefly, rabbit serum containing antibodies against the desired antigen is incubated on a thin tissue section. A second goat serum containing horseradish peroxidase coupled antibodies directed against rabbit γ-globulins localizes and amplifies the first antibody. Addition of the substrates for horseradish peroxidase, diaminobenzidine and hydrogen peroxide, leads to the formation of a dark insoluble precipitate of oxidized diaminobenzidine which can be visualized by light microscopy.

The proper controls for the system include a slide on which the anti-α-LA serum is replaced by normal rabbit serum. This control slide detects binding of the rabbit γ-globulins and cross-reactivity of tissue antigens between species. Another control slide has only the coupled goat antirabbit γ-globulin serum and detects the same types of antibody binding as above which are not in response to α-LA molecules. Because endogenous peroxidase activity can be found in milk and red blood cells, a slide which has no γ-globulin addition but is merely incubated in the substrate solution is also necessary.

The detailed conditions for these experiments include preparation of the thin tissue sections. Since the product of the reaction of horseradish peroxidase with diaminobenzidine and hydrogen peroxide is visualized by conventional light microscopy, the use of paraffin embedded thin sections is not excluded because α-LA is not antigenically altered by the embedding procedure to such an extent that it can no longer be recognized by its antibodies. The paraffin embedding process dehydrates the tissue by successive passages at 25° C for 60 minutes each in 40%, 80%, and 100% ethanol, then incubation in xylene, and finally perfusion with paraffin at 58° C for 60 minutes. To ensure good tissue preserva-

tion through the embedding process, specimens were fixed in 10% neutral buffered formalin overnight before being submitted for automated paraffin embedding. All tissue specimens were then sectioned at 5 micron thickness with a microtome, placed on glass slides and returned to the original balanced salt phase by reversing the dehydrating sequence of solutions used to embed in paraffin. The extended formalin fixation and the vigorous conditions of paraffin embedding helped to destroy the specific γ-globulin receptor binding activity of mammary epithelial cells. However, the endogenous peroxidase-like activity of hemoglobin present in red blood cells is not totally destroyed by formalin treatment or paraffin embedding. Attempts to inhibit the activity with cyanide or azide ions were unsuccessful.

The rabbit anti-α-LA sera and the normal rabbit serum were diluted 10 fold in balanced salt solution. The horseradish peroxidase coupled goat antirabbit γ-globulin was diluted to an absorbance of 0.08 at 280 nm.

The incubation conditions are similar to the immunofluorescence system and all are performed at room temperature. The immune serum and normal rabbit serum slides were incubated with their respective serum for 30 minutes. The immune serum slide, the normal rabbit serum control slide, and the goat antirabbit serum control slide, were incubated for 30 minutes with the horseradish peroxidase coupled goat antirabbit γ-globulin serum and then washed for 30 minutes in balanced salt solution. These three slides, and the diaminobenzidine control slide were then incubated in a solution of 1.4 mM diaminobenzidine, 0.0003% hydrogen peroxide (v/v), in 50 mM Tris, pH 7.4 at room temperature for 30 minutes then rinsed for 10 minutes in balanced salt solutions. Oxidized

diaminobenzidine forms an insoluble, dark brown precipitate which was visualized by conventional light microscopy. The intensity of the precipitate is rated to 0.5 units on an increasing unitless scale of 0 to 8 by double blind evaluation of two investigators. The final values reported are the average of the two blind evaluations.

Results

Rat and human tissue were tested for α-LA with the horseradish peroxidase system as shown in Table XII. The lactating rat mammary gland was taken three days after parturition and served as the positive control for the rat tissues. Preservation of tissue and cellular morphology was excellent and large amounts of secretory products were still present in the lumina. The areas of precipitate deposition were restricted to the cells lining the ascini of the lobules, with the connective stroma devoid of any precipitate. Examination of the epithelial cells with high magnification indicated only cytoplasmic precipitate which was most intense at the apical border with the lumen. The secretory products in the lumina stained heavily indicating large amounts of α-LA were present. The diaminobenzidine control slide was negative, indicating that the endogenous lactoperoxidase activity which can react with these substrates, had been denatured by the formalin and embedding processes. The normal rabbit serum control slide was slightly positive which may indicate that a small amount of residual γ-globulin receptor activity was still present after formalin fixation and paraffin embedding. The goat antirabbit γ-globulin control slide was negative. This is consistent with the data from the immunofluorescence system which indicated that the rat γ-globulin receptor on mammary

TABLE XII

EVALUATION OF α-LACTALBUMIN IN RAT AND HUMAN TISSUES
BY THE HORSERADISH PEROXIDASE METHOD

Tissue	Treatment	IS	NRS	GARγg	DAB	Net
R3230 AC	18 h NBF	3.0	0.5	0.0	0.0	2.5
R3230 AC	18 h NBF, P	3.0	0.5	0.0	0.0	2.5
Rat Mammary Gland	18 h NBF, P	5.0	0.5	0.0	0.0	4.5
13762 MAT/A	18 h NBF	0.5	0.5	0.0	0.0	0.0
DA - Fibrocystic Disease	18 h NBF, P	3.0	0.7	0.0	0.0	2.3
CO - Breast Carcinoma	18 h NBF, P	1.5	0.7	0.0	0.0	0.8
CO - Lymph Node Metastases	18 h NBF, P	1.0	0.5	0.0	0.0	0.5
RO - Ovary Carcinoma	18 h NBF, P	0.7	0.5	0.5	0.5	0.2
SW - Breast Carcinoma	18 h NBF, P	0.7	0.5	0.0	0.0	0.2
LA - Adenoma	90 d NBF, P	0.7	0.2	0.0	0.0	0.5
BT-20 Solid Tumor	18 h NBF, P	0.5	0.5	0.0	0.0	0.0
BT-20 Cell Culture	18 h NBF	0.5	0.5	0.0	0.0	0.0
CO-2 Solid Tumor	18 h NBF, P	0.5	0.5	0.0	0.0	0.0
CL-2 Solid Tumor	18 h NBF, P	0.5	0.5	0.0	0.0	0.0
ST-2 Solid Tumor	18 h NBF, P	0.5	0.5	0.0	0.0	0.0

NBF - neutral buffered formalin
IS - immune serum
NRS - normal rabbit serum
P - paraffin embedded
GARγg - goat antirabbit γ-globulin
DAB - diaminobendizene

tissue does not recognize goat γ-globulins. The net intensity of precipitate formation for the colostrum lactating rat mammary gland was 4.5. No other rat tissue was more positive.

The small R3230 AC tumor taken 10 days after injection was treated by formalin fixation for 18 hours. The tissue was then divided and part was embedded in paraffin, sectioned, and then brought back to the water phase. The other half was frozen and also sectioned on a cryostat at 5 microns thickness. The sections treated by the two methods produced identical results which verifies the antigenic stability of α-LA through the paraffin embedding process. The net intensity of precipitate formation for the R3230 AC tumor was 2.5 which is consistent with the high level of α-LA determined by the RIA.

A peritoneal aspirate of the 13762 ascites MAT/A line was air-dried on slides and fixed in formalin for 18 hours then washed in balanced salt solution for one hour. No net precipitate formation was observed. This result may be a consequence of the increased molecular dimensions of the horseradish peroxidase goat antirabbit γ-globulin complex which would decrease the antibody's ability to penetrate the cell's membrane. Some investigators have overcome this problem by splitting the antibody molecule and forming the smaller Fab-horseradish peroxidase complex which has better penetrating ability (106).

Human tissue specimens were also analyzed for α-LA by the horseradish peroxidase system. Frozen specimens were processed as described previously and evaluated for precipitate formation. The fibrocystic disease specimen from DA contained areas of epithelial cells which formed ascini that were arranged in lobular form. These regions were surrounded by large areas of fibrous stroma. The epithelial cells and

the substance contained in the small lumina were quite positive, while the stromal elements were negative. A higher background was noted on the normal rabbit serum control slide, indicating the tissue specimen may not have been totally permeated with formalin. The primary breast carcinoma from CO and the metastases present in the lymph node were both moderately positive, which supports the RIA data. The adenoma from LA had been in neutral buffered formalin for about three months before paraffin embedding and was judged only moderately positive, although the tissue contained tremendous numbers of ascini which were filled with secreted substances. Excessive formalin fixation may cause impaired detection of α-LA.

The mucinous ovary carcinoma of RO contained detectable levels of α-LA as determined by RIA. The horseradish peroxidase system confirmed the low levels of α-LA and reinforces the idea of ectopic production of proteins by some tumors (107). The cells lining the very large cysts were weakly positive, but the mucinous material filling the large cysts was essentially negative. The other breast carcinoma from SW was also weakly positive with only the epithelial cells of the ascini containing precipitate.

The second group of tumors produced in the nude mouse by subcutaneous injection of the human cell lines obtained by pleural effusion were also examined. None of the tissues were judged to be α-LA positive, which is in support of the RIA data. Hematoxylin and eosin stained paraffin sections of the nude mouse tumors revealed an extremely neoplastic condition with extensive pleomophism. No suggestive patterns of ascinar formation could be found. Slides were also prepared with the BT-20 cells and placed in neutral buffered formalin for 18 hours.

These cells which were grown in vitro culturing were also negative for α-LA.

Photomicrographs of lactating rat mammary gland treated by the horseradish peroxidase system for α-LA detection are shown in Figure 20. The immune serum slide shows areas of heavy precipitate deposition while the control normal rabbit serum slide is free from precipitate.

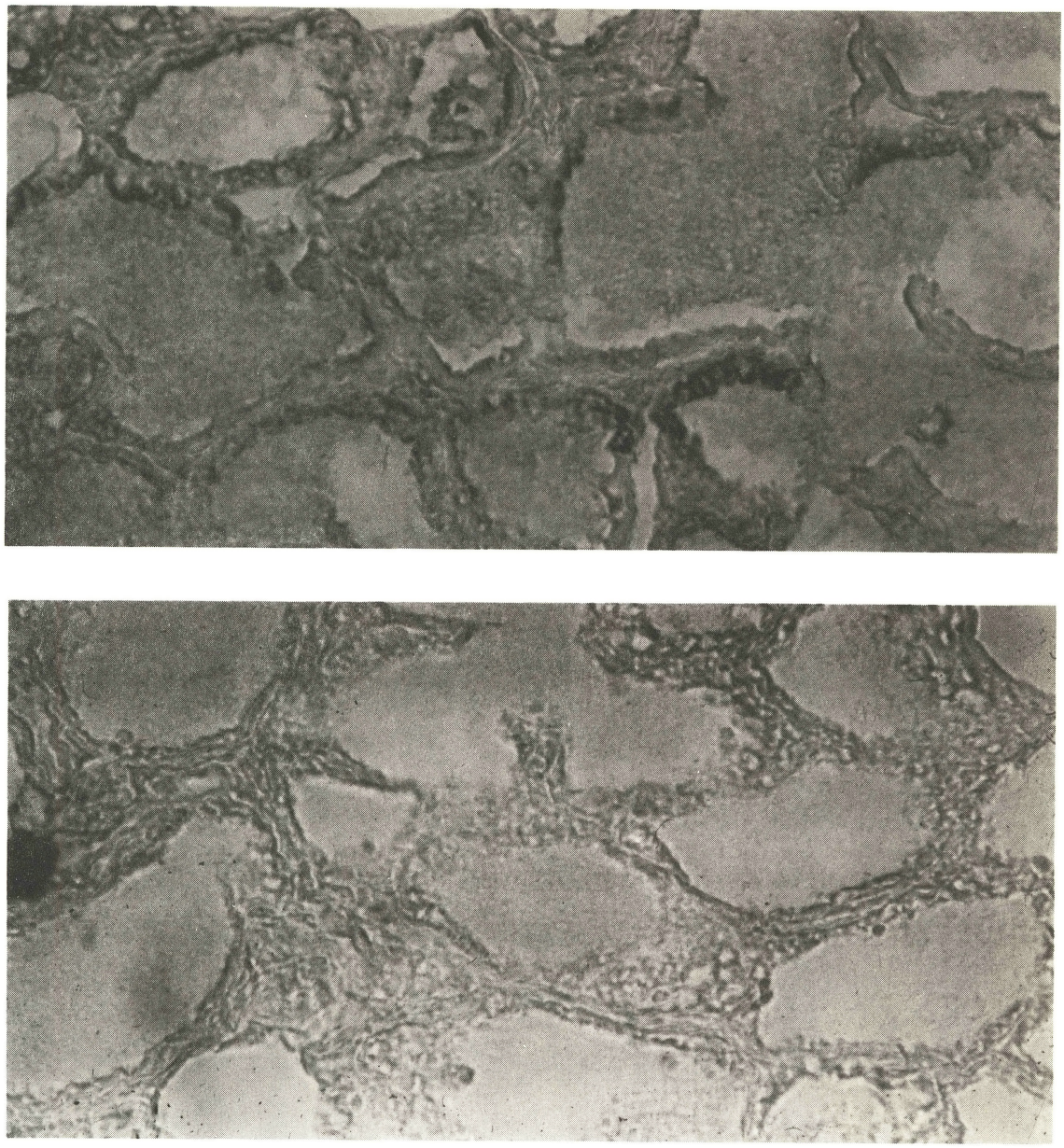

Figure 20. Photomicrographs of Lactating Rat Mammary Gland Using the Horseradish Peroxidase Method for Detection of Rat α-Lactalbumin

Sections of lactating rat mammary gland were fixed in neutral buffered formalin for 12 hours and then were embedded in paraffin. Sections five microns thick were cut and returned to the water phase. The top panel is tissue treated with rabbit antirat α-LA serum followed by peroxidase coupled goat antirabbit γ-globulins and then reacted with diaminobenzidine. The bottom panel is tissue treated with normal rabbit serum followed by peroxidase coupled goat antirabbit γ-globulins and then reacted with diaminobenzidine. (Methods).

CHAPTER VII

GAMMA GLOBULIN RECEPTORS OF MAMMARY EPITHELIAL CELLS

Materials and Reagents

Bovine γ-globulin, Cohn Fraction II (108) with electrophoretic purity greater than 99% and fluorescein isothiocyanate isomer I, were purchased from Sigma. The γ-globulin fraction of pooled normal human plasma from venous blood was purchased from Hyland.

Methods

Rat and rabbit γ-globulins were purified from pooled normal serum by the procedure of Heide and Schwick (109). Blood was collected from the animals and allowed to totally clot at 4° C overnight, then centrifuged at 5000 x g for 20 minutes at 4° C to collect the serum. Performing all steps at 4° C, the supernatant solution was made 35% (19.4 g/100 ml) saturated with ammonium sulfate and the precipitate was collected by centrifugation at 30,000 x g for 10 minutes. The pellet was resuspended in the original volumn with 20 mM Tris, pH 7.4 and the solution precipitated with ammonium sulfate and centrifuged as before. This procedure was performed a total of four times, and the final pellet was resuspended in 20 mM Tris, pH 7.4 and dialyzed exhaustively against deionized water. The dialyzed solution was centrifuged at 30,000 x g for 20 minutes and the supernatant solution analyzed for purity by

discontinuous acrylamide gel electrophoresis as described previously, and by quantitative serum analysis with cellulose acetate strips (91). Both systems indicated the commercial human and bovine γ-globulins had greater than 99% purity. The rabbit and rat γ-globulins prepared by the above procedure contained greater than 96% γ-globulins with 2% β-globulins and 2% α_2-globulins. No serum albumin could be detected in any γ-globulin sample.

The purified γ-globulin fractions were conjugated with fluorescein isothiocyanate by the method of Marshall et al. (110). The γ-globulin fractions were suspended in 15 mM NaCl, 50 mM $NaHCO_3$, pH 9 to a final protein concentration of 10 mg/ml. Performing all steps at 4° C, 50 µg of fluorescein isothiocyanate were added for each milligram of γ-globulin, and the solution gently stirred for 18 hours. The conjugated γ-globulin fraction was again made to 35% (19.4 g/100 ml) saturation with ammonium sulfate and the precipitate collected by centrifugation at 30,000 x g for 10 minutes and resuspended in the original volume with 20 mM Tris, pH 7.4. This procedure was repeated a total of four times. The final pellet was resuspended at a 16 mg/ml level with 10 mM phosphate, 0.14 M NcCl, 4 mM KCl, pH 7 and dialyzed extensively against the resuspending buffer until the dialysate was visually frre of fluorescence when exposed to ultraviolet light. The dialyzed solution was centrifuged at 30,000 x g for 20 minutes and the supernatant solution stored at -20° C.

Tissue samples were prepared by quickly freezing the excised tissue specimens in liquid nitrogen, then cutting sections as thinly as possible with the -20° C cyrostat. The tumor sections were cut at 5 microns and the lactating rat and rabbit mammary glands were cut at 12 microns. The

tissue sections were air-dried on the slides at room temperature for 30 minutes, and then placed in 10% neutral buffered formalin solution (10% (v/v) formaldehyde, 50 mM phosphate, pH 7.0) for 5 minutes or 18 hours at room temperature. The sections were washed in balanced salt solution (0.14 M NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 0.17 mM NaH_2PO_4, pH 7.2) for one hour and then used in the experiments.

The objectives of the following experiments were (1) to demonstrate the presence of γ-globulin receptors on mammary epithelial cells and (2) to determine the ability of these receptors to recognize γ-globulins from other species. Performing the experimental procedure at room temperature, sections from a particular tissue were incubated for one hour with solutions of its fluorescein labeled γ-globulin which were diluted with a 50 fold greater level of unlabeled γ-globulins from the other species. The slides were washed for one hour in balanced salt solution, mounted in glycerin and judged by double blind evaluation of two investigators on the Zeiss ultraviolet microscope using an increasing unitless scale of 0 to 8. For example, 3 day lactating rat mammary gland was removed, frozen, sectioned, and fixed as described. The fluorescein labeled rat γ-globulins were diluted 10 fold with 0.1 M KCl, 20 mM Tris, pH 7.4 containing 0.1% (w/v) bovine serum albumin. The solution was, therefore, 1 mg fluorescein labeled rat γ-globulin/ml and 1 mg bovine serum albumin/ml. The fluorescein labeled rat γ-globulins were also diluted 10 fold with purified unlabeled γ-globulins from other species at 50 mg γ-globulins/ml in 0.1 M KCl, 20 mM Tris, pH 7.4 containing 0.1% bovine serum albumin. Five slides were incubated for one hour at room temperature with the rat fluorescein labeled γ-globulins diluted with the buffer only, or the buffer with excess unlabeled rat,

rabbit, human, or bovine γ-globulins. The slides were then washed for one hour in balanced salt solution, mounted, and evaluated as described before. For each different tissue the same basic procedure used. For example, when examining the rabbit lactating mammary gland, the fluorescein labeled rabbit γ-globulins were diluted 10 fold with the buffer or with the buffer containing 50 fold greater concentration of unlabeled rabbit, rat, human, or bovine γ-globulins.

Results

Three rat mammary tissues were analyzed for the presence and specificity of the γ-globulin receptors. As shown in Table XIII, the colostrum lactating rat mammary gland had a high level of γ-globulin receptors as indicated by the 3.3 net displacement of fluorescence between the slides incubated with and without excess unlabeled rat γ-globulins. As predicted by the previous immunofluorescence data, rabbit γ-globulins were also successful in binding to the γ-globulin receptors and produced a displacement value of 3.0. Unlabeled bovine and human γ-globulin were not very effective competitors with rat γ-globulins for the rat mammary gland γ-globulin receptors. They produced displacement values of only 1.0 and 1.3 respectively. This also supports the data from the previous fluorescence study where bovine γ-globulins were not effective at reducing the fluorescence of the normal rabbit serum control slide.

The R3230 AC adenocarcinoma indicated a slightly higher level of γ-globulin receptors than the lactating mammary gland with the same trends of displacement by γ-globulins from other species. However, the differences between the values for displacement by unlabeled γ-globulins

TABLE XIII

EVALUATION OF γ-GLOBULIN BINDING TO MAMMARY EPITHELIAL CELLS OF THREE SPECIES

Rat Tissue	FL-Rat γg	FL-Rat γg + Rat γg	FL-Rat γg + Rabbit γg	FL-Rat γg + Bovine γg	FL-Rat γg + Human γg	Auto
Rat Mammary Gland	4.0	0.7	1.0	3.0	2.7	0.5
R3230 AC	4.7	0.5	1.0	1.5	1.7	0.0
13762 MAT/A	2.5	1.0	1.0	1.5	1.5	0.7

Human Tissue	FL-Human γg	FL-Human γg + Rat γg	FL-Human γg + Rabbit γg	FL-Human γg + Bovine γg	FL-Human γg + Human γg	Auto
CO - Breast Carcinoma	3.5	1.0	2.0	0.5	0.5	0.0
CO - Lymph Node Metastases	3.5	0.7	2.5	0.5	0.5	0.0
DA - Fibrocystic Disease	2.0	0.5	0.5	0.5	0.2	0.0

Rabbit Tissue	FL-Rabbit γg	FL-Rabbit γg + Rat γg	FL-Rabbit γg + Rabbit γg	FL-Rabbit γg + Bovine γg	FL-Rabbit γg + Human γg	Auto
Rabbit Mammary Gland	7.0	2.0	1.5	2.0	1.5	0.5

FL-γg - fluorescein labeled γ-globulin from indicated species

Auto - autofluorescent control

from the different species was not as dramatic as for the lactating mammary gland. The ascites 13762 MAT/A line cells had rather low levels of γ-globulin receptors with the same trends seen for the lactating mammary gland. Again however, there was not a large difference between the displacement values for unlabeled γ-globulins from the other species. This increased degeneracy of the γ-globulin receptors of malignant mammary tissue may be a reflection of the malignant process.

Human tissues were also investigated and the epithelial cells of the various mammary diseases possessed γ-globulin receptors. The ability of the γ-globulin receptors to recognize γ-globulins from other species was generally reversed from the rat mammary tissues. The excess rat and rabbit unlabeled γ-globulins were not as effective in competing for the γ-globulin receptors as were the bovine and human γ-globulins. However, the trends were not always unequivocal and more tissues should be examined to determine if a general trend is apparent.

Rabbit lactating mammary gland was investigated and it contained a very high level of γ-globulin receptors. The data indicated these receptors recognize rabbit and human γ-globulins equally well and also have rather good ability to recognize rat and bovine γ-globulins.

To ensure that the test system was not subject to large nonspecific protein binding effects, the following experiment was performed. The colostrum forming rat mammary gland sections were incubated with solutions of fluorescein labeled rat γ-globulins that were diluted 10 fold in 0.1 M KCl, 20 mM Tris, pH 7.4 containing bovine serum albumin at different levels. As shown in Table XIV, incubation of the fluorescein labeled rat γ-globulin in solutions of 0 to 5% (w/v) bovine serum albumin did not cause any detectable decrease of fluorescence. This suggests

TABLE XIV

EFFECT OF BOVINE SERUM ALBUMIN ON γ-GLOBULIN BINDING TO RAT MAMMARY EPITHELIAL CELLS

mg/ml of Bovine Serum Albumin with Fluorescein Labeled Rat γ-globulin	Fluorescence of Tissue Section
0.0	3.5
1	3.5
10	3.5
50	3.5
80	3.2
autofluorescence	0.0

that there is essentially no nonspecific binding of the γ-globulin to mammary epithelial cells, and that the observed fluorescence is due to specific and selective binding of γ-globulins. Further, in no tissue did the supporting stroma take part in the specific binding of γ-globulins. The fluorescence was limited to the epithelial cells lining the ducts.

CHAPTER VIII

DISCUSSION AND SUMMARY

To date, the biochemical level of differentiation of breast tumors has not been investigated using a reliable marker protein that is hormonally controlled and specific for mammary epithelial cells. Because the enzymatic measurements of α-LA is often prohibited by competing enzyme systems, radioimmunoassays for human and rat α-LA were developed. This required ultrapure α-LA which was obtained by a modification of the Schmidt (89) procedure. By performing two ammonium sulfate precipitation steps instead of one, more contaminating proteins were removed early in the purification. Removal of the casein micelles by centrifugation at 100,000 x g for one hour proved satisfactory. The P-150 column was very effective at resolving the α-LA activity from the remaining higher molecular weight contaminants. The DEAE-32 column removed the remaining minor contaminant and pure human α-LA was obtained. Antisera to rat α-LA and human α-LA were prepared from rabbits and their titers were exceptionally high. Double diffusion analysis for cross-reactivity of both antisera indicated that antirat α-LA antibodies did not cross-react with any other α-LA except human. The antihuman α-LA antibodies displayed a time dependent cross-reactivity to α-LAs from other species which was absent in sera drawn three weeks after injection but present in sera drawn five weeks after injection. The monospecific nature of the antisera was demonstrated by a single band in double diffusion tests

against their whey fraction. Also, immunoelectrophoresis against the pure α-LA and their whey fraction produced a single band with antihuman α-LA serum and a spurred band for antirat α-LA serum. This observation for rat α-LA is a reflection of the known charge isomers of rat α-LA.

The optimal conditions for both RIAs were determined. α-Lactalbumin with a specific activity of about 80 μCi/μg is required and can be obtained by the iodochloride method, chloramin-T method, or lactoperoxidase method. Free and antibody bound ^{125}I-α-LA are quantitatively and efficiently separated by using polyethylene glycol precipitation of the γ-globulin α-LA complex which can be collected by filtration on 0.45 μ cellulose acetate filters. The effects of pH, ionic strength, and protein on the RIAs were investigated and optimized conditions were determined to be pH 7.4, 0.1 M KCl, and 0.1% bovine serum albumin. It is important to note that protein levels greater than 10 mg/ml can cause false positive values of α-LA. Samples must meet the requirements of pH between 6 and 8.5, ionic strength between 4 and 22 m mho, and protein concentration between 1 and 10 mg/ml to achieve reliable answers. The temperature and sequence of addition of reactants to the RIAs can increase the limit of detection about two times. This was accomplished by incubation at 4° C and sequential addition of antiserum and unlabeled α-LA with 12 hours of incubation followed by ^{125}I-α-LA addition and 12 hours additional incubation. These techniques generated standard curves with limits of detection at 30 pg α-LA in the assay or 0.3 pg α-LA/μg and 75 pg α-LA/ml. There was no displacement by homogenates of normal rat tissues or normal human tissues in the RIAs.

Rat model tumors for human neoplasms were analyzed for α-LA and a wide range of levels was found. The group of tumors histologically

designated as adenocarcinomas ranged from 1.21 ng α-LA/μg protein to 0.096 ng α-LA/μg protein. While the SMT-2A breast carcinoma had 0.0084 ng α-LA/μg protein yet was described histologically as poorly differentiated. This indicates that estimation of differentiation by visual examination of tissue sections cannot reliably distinguish tissues except in some of the extreme examples. The three ascites lines still retained the ability to synthesize α-LA although at lower levels than the parent tumor. This may be a reflection of the growth conditions inherent with the ascitic state and should be kept in mind when comparisons are made to the biochemical status of normal mammary cells.

Sera from rats bearing tumors were analyzed and a correlation was found between the tumor size, as measured by the time after transplantation, and the serum level of α-LA. The 13762 ascites MAT/A line produced detectable levels almost immediately and eventually rose to about 100 ng α-LA/ml serum at about ten days. The R3230 AC tumor had undetectable levels until about two weeks after transplantation. Then serum α-LA levels began to rise and eventually reached near lactating serum levels of about 300 ng α-LA/ml serum. The quick rise of α-LA in the sera from rats bearing the ascites line is probably due to the immediate growth of the injected cells and the rapid equilibrium of compounds in the peritoneal cavity with the general body circulation. The transplanted R3230 AC tumor, on the other hand, does not develop palpable tumor masses until about seven to ten days after transplantation. This initially inhibited growth rate probably is due to an immunological response of the host, which is eventually overcome by the tumor and then α-LA appears in the blood. These observations suggest that it would be theoretically possible to detect and

monitor mammary tumors by analysis of serum for α-LA.

To estimate the percentage of human breast carcinomas that synthesize α-LA and their range of values, ten breast carcinomas were analyzed. Seven were found to contain detectable levels with a range of 0.4 to 22.0 pg α-LA/μg protein. Two cases of metastases to the associated lymph node from primary tumor positive for α-LA were analyzed and both synthesized α-LA at levels near or exceeding the parent tumor. These data suggest that the metastasized cells still retain the original level of differentiation of the parent tumor cells.

Seven cases of benign fibrocystic/fibroadenoma disease were analyzed and all seven were positive for α-LA. Several reached very high levels of α-LA, the range being from 116 to 1.3 pg α-LA/μg protein. This observation would indicate the fibrocystic/fibroadenoma disease should generally be considered as a more differentiated disease state than a valid carcinoma. Since current theories consider cancer as a dedifferentiation process, these diseases could be considered as candidates for valid precancerous states.

Analysis of malignant tumors from the lung and pancreas were negative for α-LA, as were the benign myelofibrosis of the spleen and the male gynecomastia. One case of an ovary carcinoma contained α-LA. This probably is due to ectopic production of α-LA.

Presurgery sera from breast carcinoma patients were assayed and 10 of the 35 had detectable levels of α-LA which had an average value of 10.2 ng α-LA/ml serum. All of the postsurgery sera from the patients with positive presurgery were 0.0, indicating removal of the tumor removed the source of α-LA production. All control male and female sera were also 0.0. These data suggest that an abnormal level of serum

α-LA may indicate a breast lesion. These values are low compared to the levels reported in the rat tumor system and are probably due to the very large size of the rat tumors compared to the total body weight. In humans, a tumor would probably be detected by conventional means before it reached the proportional size obtained in rats. The work of Kleinberg (67) indicated only 5 of 44 patients with metastatic breast carcinoma had detectable levels of serum α-LA with a range of 1 to 12 ng α-LA/ml and a mean value of 6 ng α-LA/ml. In addition he also reports 9 of 22 normal males and 15 of 33 normal females had serum α-LA levels with mean values of 5.83 ng α-LA/ml and 6.42 ng α-LA/ml respectively. It is difficult to accept that α-LA could appear in the serum of men even with gynecomastia. Hendrick and Franchimont (73) have reported that casein is found in the sera of 80 percent of patients with metastatic breast carcinoma. However, their levels are 1000 times greater than the levels of α-LA reported here. Also, they reported high levels of casein in sera from patients with lung and gastric cancers. Most significantly, they have been unable to establish any correlation between the presence of casein in the serum and the histological nature of the tumor. In view of the polymorphism of human caseins, they probably are not a reliable marker antigen for human mammary dysplasia.

Because the serum levels of α-LA from presurgery breast cancer patients were low, it was of interest to determine the rate of clearance of α-LA from blood. The half life was determined to be 3.5 hours which is longer than polypeptide hormones like insulin, which has a 1.5 hour half life (111), but shorter than normal serum proteins like transferrin which has a half life of 7 to 10 days (112) or immunoglobulin G which has a half life of 18 to 23 days, or immunoglobulin E which has a half

life of 2 to 4 days (113). This rapid clearance of even large levels (6 mg) of α-LA probably helps to maintain very low blood levels except in periods of relatively intense secretion. The clearance rate from blood for caseins has not been determined and would be of interest in view of the high levels reported in sera from breast carcinomas.

The organs primarily responsible for clearance of α-LA from blood are the liver and lungs, with a large contribution by capillary beds such as found in muscle. Because no increase in trichloracetic acid soluble material appears after injection of labeled α-LA, there probably is no immediate degradation with release of fragments to the serum.

Purported human mammary carcinoma cell lines obtained by pleural effusion were assayed for α-LA and all were negative, even if injected into athymic mice and regrown as solid tumors. These results raise serious questions as to the validity of assuming that the cells comprising a pleural effusion of a breast carcinoma contain malignant differentiated mammary cells. The question must be asked whether the cells have lost the ability to make specialized milk proteins as a consequence of cell culturing conditions, or whether the original cell population ever contained differentiated mammary cells at the start of culturing. One report (65) claims to have successfully cultured a pleural effusion from a mammary carcinoma that synthesized 0.155 ng α-LA/μg protein, which is significantly higher than the levels of the rat 13762 ascites lines (0.02 ng α-LA/μg protein).

Two methods for detecting α-LA in situ were developed: the immunofluorescence system and the horseradish peroxidase system. These methods

qualitatively confirmed the RIA results. Paraffin embedded tissue sections developed severe autofluorescence, which restricted immunofluorescence studies to fresh frozen samples. The horseradish peroxidase method, however, worked best on paraffin embedded tissue samples. Cases from the surgical pathology files were investigated in an effort to determine whether certain diseases were actually related to breast cancer, and to determine their degree of differentiation.

Areas of cells within tumors were judged by immunofluorescence to produce different levels of α-LA, indicating that tumors probably contain populations of cells with varying levels of differentiation. Homogenization would indicate an average value of α-LA production for a particular area of a tumor. Bussolati and Pich (78) analyzed human breast diseases for caseins using immunofluorescence and peroxidase methods. Their data indicated most breast carcinomas produced caseins; however, the sebaceous glands also stained. This may be a reflection of endogenous peroxidase activity and should be clarified before valid conclusions could be drawn. The use of casein as the antigen marker for histochemical studies suffers from the same drawbacks as mentioned before.

In the course of developing the immunofluorescence system, specific γ-globulin receptors were detected on mammary epithelial cells. These receptors could be blocked by γ-globulins to which the fluorescein labeled goat antirabbit γ-globulins did not cross-react. Also they could be chemically modified by formalin fixation which resulted in the loss of their biological activity. By directly labeling purified γ-globulins with fluorescein and incubating them with excess unlabeled γ-globulins, it was possible to determine the degeneracy of the γ-globu-

lin receptors on mammary epithelial cells from different species. The γ-globulin receptors of rat mammary tissue effectively recognized only rat and rabbit γ-globulins. The γ-globulin receptors of human and rabbit mammary tissue recognized both human and rabbit γ-globulins with substantial binding of the bovine and rat γ-globulins also.

These data would indicate that clinical diagnosis of breast diseases could be aided by serum analysis and determination of the lesion's degree of differentiation. All of the purported human mammary cell lines that were assayed did not contain detectable levels of α-LA, and the validity of the pleural effusion technique should probably be re-evaluated. The use of the horseradish peroxidase technique for detecting α-LA has opened tissue files of pathology departments for examination of large numbers of disease states. Further investigation on the specificity of γ-globulin receptors of normal and malignant states may lead to a clinically applicable method of selecting malignant cells in vivo.

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