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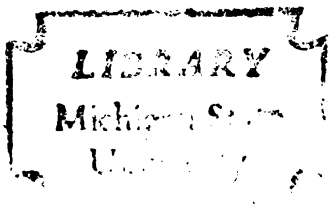

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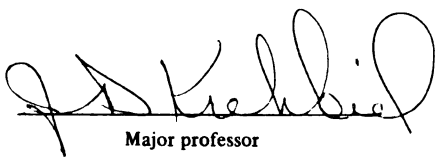
THE UNIQUE ASSOCIATION OF ALANINE AMINOTRANSFERASE WITH THE TRANSPLANTABLE R3230AC-L ADENOCARCINOMA IN THE FISCHER RAT

presented by

Christine Cecilia Hall

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Pathology



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THE UNIQUE ASSOCIATION OF ALANINE AMINOTRANSFERASE WITH THE TRANSPLANTABLE R3230AC-L ADENOCARCINOMA IN THE FISCHER RAT

By

Christine Cecilia Hall

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pathology

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ABSTRACT

THE UNIQUE ASSOCIATION OF ALANINE AMINOTRANSFERASE
WITH THE TRANSPLANTABLE R3230AC-L ADENOCARCINOMA IN THE FISCHER RAT

BY

Christine Cecilia Hall

The Research 3230 Adenocarcinoma, Lung subline (R3230AC-L), in the Fischer 344 rat is a transplantable mammary tumor. When placed beneath the kidney capsule, it metastasized readily to the lung causing asphyxiation and death within 6 weeks of implantation. Associated with the growing tumor load was an elevation of serum levels of alanine aminotransferase (ALT), an enzyme considered specific for indicating heaptic damage. Grossly, microscopically and ultrastructurally, there was no indication of hepatic change or damage in tumor-bearing rats. Further serum clinical chemistry tests also confirmed the absence of hepatic damage.

The R3230AC-L tumor, metastases and liver from tumor-bearing rats were homogenized then analyzed for ALT activity along with serum from the same rats. Serum and liver homogenates from control rats were also analyzed for the enzyme. Tumor tissue contained approximately one fourth the activity of ALT found in liver tissue. Tumor tissue by weight was approximately 3 times the liver. Despite the lower activity of enzyme per gram of tumor tissue, the R3230AC-L tumor was considered responsible for the increased serum levels of ALT based on the greater amount of tumor tissue and the fact that, being necrotic, the tumor could have been leaking enzyme constantly into the circulation.

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Starch gel electrophoresis was performed to compare ALT from the primary tumor, metastatic nodules, liver and serum of tumor-bearing rats and liver and serum of control rats. No differences were seen. It was concluded that the enzyme in the tumor tissues was very similar, if not identical, to the ALT produced by the liver.

These results have identified and characterized a new research tool for the study of neoplastic disease. The association of ALT with the R3230AC-L tumor could be utilized as an indicator system for the estimation of tumor development in tests of anti-tumor agents. In addition, it was suggested that a similar association between certain breast cancers and ALT might occur in man and could serve as a diagnostic or prognostic test.

Dedicated to my husband, Alec.
I could not have done this without him.

ACKNOWLEDGEMENTS

The author wishes to express sincere gratitude to the members of her graduate committee: Dr. J. D. Krehbiel, Dr. T. J. Kakuk, Dr. R. F. Langham, Dr. H. Tvedten and Dr. D. Greenbaum. All of these people have provided invaluable guidance and encouragement in the preparation of this thesis. Sincere thanks and appreciation go to Dr. Krehbiel, her major professor, who offered direction, guidance and so much of his time from the very beginning of the graduate program. Sincere gratitude is extended to Dr. Kakuk who provided insight and the R3230AC-L tumor for this research and who, together with Dr. Krehbiel, secured the financial support needed.

The author also wishes to sincerely thank The Upjohn Company who provided the financial support for much of the author's graduate program and whose people were most helpful. Time and space do not allow for individual recognition of all those people, but their assistance will always be gratefully remembered.

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INTRODUCTION

Cancer is a very broad term for literally hundreds of diseases involving a number of different organ systems. To the scientist the word, cancer, brings to mind an intricately designed puzzle with pieces which must be placed together in the right proportion, in an exacting order, at precisely the right time in order to produce the outward picture of uncontrolled growth of cells.

One of the most valuable tools for solving that puzzle is an animal model. But to be useful, the animal model must be studied in great detail from every angle and at every level even down to its molecular level. Only after such detailed research will some of the pieces fall into place bringing scientists closer to answering the many questions regarding malignant transformation of cells.

The research herein was aimed at providing some of those pieces in the cancer puzzle, more specifically relating to mammary cancer, through studies of an animal model. The animal model was the Research 3230 Adenocarcinoma (lung subline) in the Fischer 344 rat. While some questions were answered by this work, as happens quite frequently, new questions arose.

LITERATURE REVIEW

The R3230 Mammary Adenocarcinomas

History

The genealogy of the Research 3230 Adenocarcinoma-Lung (R3220AC-L) mammary adenocarcinoma can be traced back to the R3230 tumor which arose spontaneously in a 322-day old Fischer 344 breeding female rat (Figure 1). Dunning (1960) described the tumor as a papillary cystadenocarcinoma. It was maintained and perpetuated by periodic subcutaneous transplantation. Hilf et al (1963a, 1963b) continued the study of the R3230 tumor as a possible animal model of certain hormone responsive breast cancers in man. They initially worked with the R3230 tumor and a faster growing more responsive subline, the R3230-S. Both of these lines appeared to be consistant in their biochemical behavior and morphologic characteristics even after many transplant generations. This consistancy is a highly desirable characteristic in research tumors.

Another subline developed at about the same time was the R3230AB (Hilf et al 1964). This neoplasm grew rapidly and had the unique characteristic of lactating in response to estrogen treatment. Histologically it was characterized by the presence of extensive stromal elements which had a propensity for sarcomatous development with successive transplantations.

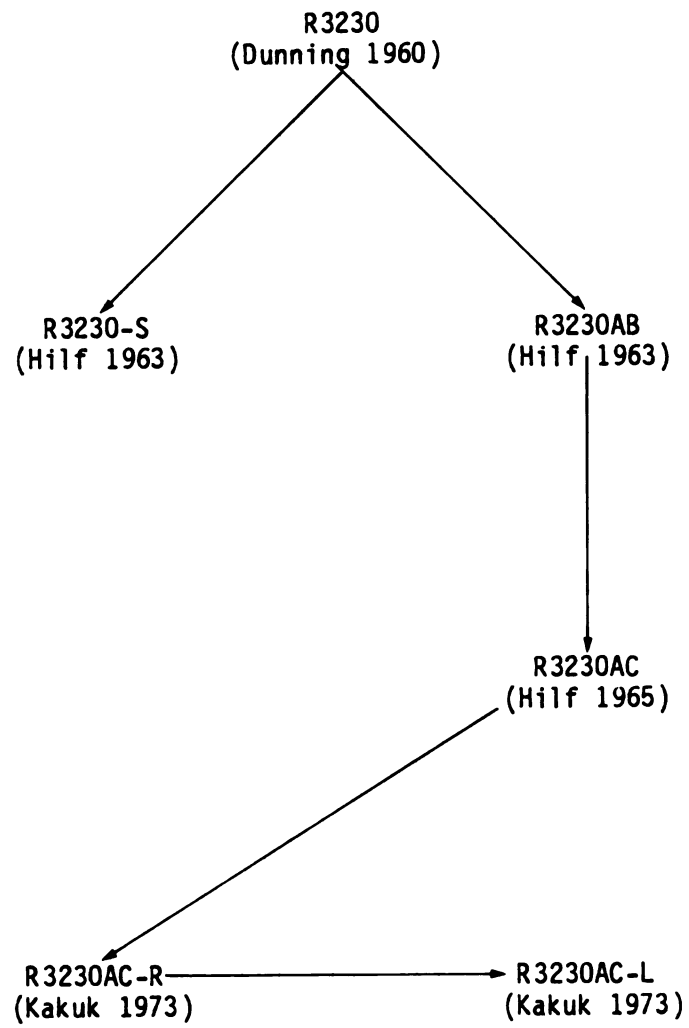

Figure 1. Genealogy of the R3230AC-L tumor.

From the R3230AB line came the R3230AC line (Hilf et al 1965a). This new line arose as a slow growing variant during the 18th transplant generation of the R3230AB tumor. The R3230AC line contained more glandular epithelial elements than the parent line and had many interesting characteristics which made it the most valuable research tumor of the group. The R3230AC also correlated well with certain breast cancers occurring in man with respect to RNA/DNA ratio, lipid content and enzyme patterns. Since 1965 most of the research efforts with the R3230 tumor groups have concentrated on the R3230AC subline.

Kakuk (unpublished data 1973) implanted the R3230AC tumor beneath the kidney capsule to study the mechanisms of metastasis and to develop an animal model to test antitumor agents. Previous researchers had transplanted the tumor subcutaneously only. Tumors placed in this location did not metastisize until approximately 13 weeks post-transplantation. On the other hand, pulmonary metastasis from the kidney implant of the R3230AC tumor occurred within 6 weeks. This intrarenally transplanted tumor line was called the R3230AC-R line (Research 3230 Adenocarcinoma-Renal). Because this line had a tendency to become sarcomatous, it was discontinued. Still another line was developed by Kakuk by taking the metastatic lung nodules and using them for intrarenal transplantation. It was designated the R3230AC-L line. This line has been maintained to the present by successive transplantations at approximately 5 week intervals. This was the tumor line selected for the research reported herein.

Gross Characteristics

There were very few descriptions of any of the R3230 tumors in the literature. The R3230AB tumor was described as granular, lumpy and slightly yellow (Hilf et al 1964). The R3230AC tumor was described as encapsulated, yellow, very soft and spongy with glandular puff-like structures which, when cut, exuded a white fluid (Hilf et al 1965a).

Histologic Characteristics

Dunning (1960) categorized the R3230 tumor in intact female rats as a papillary cystadenocarcinoma. In male rats the tumor had a tendency to develop areas of squamous metaplasia. Characteristic morphologic changes occurred in all the sublines after hormone administration to the tumor bearing rats. Some of these changes will be discussed further under the section dealing with hormone treatment.

The R3230AB line, according to Hilf et al (1964), had 2 distinct morphologic elements, epithelial and connective tissue. These were present in varying proportions with varying degrees of malignancy in each. If extra care was not taken to transplant only glandular elements, the tumor had a tendency to progress to a sarcoma.

Hilf et al (1965a) classified the R3230AC tumor as a mammary adenocarcinoma consisting primarily of epithelial cells. Only small amounts of stromal elements were present in contrast to the R3230AB tumor. Secretory activity in the R3230AC was indicated by the cellular uptake of oil red O.

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Tumor Milk

The white fluid secreted by the R3230AC in response to estrogen treatment was analyzed and examined by several investigators. This fluid was compared to milk obtained from actively lactating Fischer rats. Hilf (1967) concluded that this fluid from the tumor was similar to normal rat milk. The tumor milk contained a sugar chromatographically identical with the lactose present in rat milk but at a percentage of 0.06 which is much lower than the 3% of normal milk. The fatty acid content of the tumor milk consisted of a higher concentration of short chain fatty acids compared to normal milk. The total protein content of the tumor fluid was much higher than that of normal rat milk, but there were protein components in the tumor "milk" which were electrophoreticly very similar to the casein and whey proteins of normal milk.

McGuire (1969) demonstrated the presence of the enzyme lactose synthetase in the tumor tissue with activity normally found in the rat mammary gland only during late pregnancy and early lactation. He concluded that the tumor could be considered in a functional state of differentiation comparable to the normal mammary gland during late pregnancy and early lactation.

Turkington (1970) demonstrated a uniformity of function in all of the R3230AC tumor cells. Through the cellular localization of casein and α-lactalbumin, he concluded that all of the tumor cells were equally functional in the production of the tumor milk. This, according to Turkington, would also suggest that the secretory stimulation in the tumor by certain hormones is due to an increased rate of synthesis per

cell rather than an increased percentage of cells engaged in milk synthesis.

Plasma Membrane and Membrane Receptors

Several investigators conducted studies designed to compare the constituents of the tumor cell plasma membrane with normal tissues (Carraway et al 1974 and Shin et al 1975). Polypeptide and enzyme analysis revealed only minor differences between the tumor and normal tissue. In the case of membrane glycoproteins, however, the differences were more significant. Harmon and Hilf (1976) utilized in vitro studies to identify the nature of insulin receptors in the tumor. They concluded that the insulin receptor was similar to that of normal tissue which would suggest neoplastic transformation does not necessarily alter the character of the insulin receptors. Shafie et al (1977) found that insulin binding was influenced by ovarian hormones. There was increased insulin binding in tumors from ovariectomized rats. This binding was decreased when estrogens were administered. Turkington (1974) confirmed the existence of specific prolactin binding sites on the plasma membrane.

Mitochondria

Senior et al (1975) compared mitochondria from the R3230AC tumor with mitochondria from normal mammary glands of both pregnant and lactating rats. No significant differences were observed in mitochondrial enzymes activities or cytochrome a content. The inner membrane carriers for phosphate, dicarboxylic acids and tricarboxylic acids were functionally similar in the tumors and normal tissue

mitochondria. There were no major differences in the inner mitochondrial membrane architecture. The differences noted were a lower yield of mitochondria from the tumor tissue and subtle differences in the properties of the adenine nucleotide carrier and ATPase at the inner mitochondrial membrane. The adenine nucleotide carrier had an above normal V_{max} for ADP transport and a below normal KmADP. Based upon electrophoretic work, the tumor ATPase was suspected of being slightly different structurally from normal.

Hormonal Treatment Effects

Several researchers studied the growth response of the tumor after hormone treatment of the tumor bearing rat. Estrogen decreased the neoplastic growth rate regardless of when hormone treatment was initiated after tumor implantation. However, the R3230AC tumor was more sensitive to estrogen during the early stages of growth. In addition, estrogen treatment always induced the secretion of the milk-like fluid (Hilf et al 1965a). Klein and Loizzi (1977) reported in their electron microscopic observations of estrogen treated tumor tissues that the tumor cells appeared well differentiated resembling the cells of an actively lactating mammary gland.

Tumor response to testosterone depended upon the time that hormone therapy was initiated relative to the time of tumor implantation (Hilf et al 1965a). Tumor growth was inhibited when testosterone was administered on the first day after tumor implantation. If testosterone was administered on the 30th day after implantation, the growth was enhanced. Histologic examination of testosterone treated tumors revealed more compact and nonsecretory appearing epithelial cells.

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Fluphenazine HCl was used to induce endogenous secretion of prolactin in tumor bearing rats and thereby to test the effect of prolactin on the R3230AC tumor (Hilf et al 1971). The results were a decrease in growth of the tumor while normal mammary glands were stimulated. A secretory response to prolactin by the tumor tissue was observed in this study as well as studies by McGuire (1969) and Smith et al (1977).

Cohen and Hilf (1975) studied the effects of insulin on the R3230AC tumor. They found that the tumor was not dependent upon the presence of insulin. Insulin could inhibit the growth of the tumor and insulin plus estrogen treatment appeared additive in inhibition of tumor growth.

The effect of hormonal treatments on the nucleic acid content of the R3230AC adenocarcinoma was also measured. In the studies of Hilf and coworkers (1965a) the observed alterations were small. Estrogen, when administered alone or in combination with prolactin, produced an increased RNA/DNA ratio due to decreased amounts of DNA. Testosterone produced a similar but smaller response. In a study using endogenous prolactin there was a dose-related decrease in the DNA content of the carcinoma resulting in a dose related increase in the RNA/DNA ratio (Hilf et al 1971).

Metabolic alterations due to hormone administration were assessed by measuring changes in enzyme activities within the tumor tissue. The effects of estrogen were reported in several studies (Hilf et al 1965a, Hilf et al 1966a, and Ringler and Hilf 1974). The activities of glucosephosphate isomerase, glycerolphosphate dehydrogenase and isocitric dehydrogenase decreased. There was increased activity of glucose 6-phosphate dehydrogenase, malate dehydrogenase and phosphoglucomutase

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compared to normal mammary tissue. The actual amounts of glucose 6-phosphate dehydrogenase, malate dehydrogenase and isocitric dehydrogenase were higher in the untreated tumor tissue. The magnitudes of the enzyme responses to hormonal stimulation, however, were much less in the tumor tissue compared to normal mammary tissue. Richards and Hilf (1972) looked at isoemzyme changes in response to estrogen treatment. Their results agreed with the previous research in that the magnitude of response was much less in the tumor tissue. It was noted that the increased activities in both tissues were due to the same isoenzymes. Estrogen caused increased activities of glucose 6-phosphate dehydrogenase-2, lactate dehydrogenase-4, and lactate dehydrogenase-5 in the normal mammary gland and in the R3230AC tumor. Testosterone depressed glucose 6-phosphate dehydrogenase and malic enzyme activities. Prolactin treatment produced an increase in the activities of glucose 6-phosphate dehydrogenase, malate dehydrogenase, phosphoglucomutase and aspartate aminotransferase (Hilf et al 1971). Insulin treatment decreased the activities of glucose 6-phosphate dehydrogenase and pyruvate kinase (Cohen and Hilf 1975). The prevention of hormone-induced changes in enzyme activities by the concomitant administration of either actinomycin D or actidione, led Hilf et al (1965b, 1968) to conclude that the estrogen and androgen-induced responses in enzyme activities reflected protein synthesis de novo.

Hormone induced changes in the tumor lipids were analyzed by Zalenski and Hilf (1974). Estrogen caused a dose related increase in free fatty acids and triglycerides due to increases in short to medium chain fatty acids. There was also a decrease in longer chain fatty acids. There was no apparent effect on the levels of cholesterol.

These results were consistant with earlier findings (Hilf et al 1966a). Prolactin treatment caused a small increase in the levels of free fatty acids and triglycerides in the R3230AC tumor due primarily to an increase in the percentage of short chain fatty acids in these lipid fractions. In a later study Hilf et al (1971) reported decreased levels of cholesterol after prolactin treatment. Insulin had no effect on tumor lipids.

Estrogen Receptors

Witliff et al (1972) demonstrated the presence of cytoplasmic macromolecular receptors for ^{3}H-estradiol-17β on the tumor cells. These receptors were similar to those of the lactating mammary gland in degree of hormonal specificity and affinity. In addition, in the cytosol of the tumor cells there were significant quantities of the receptors with binding capacities approaching those of the lactating mammary gland.

Endocrine Organ Ablation and Tumor Growth

Ovariectomy or orchiectomy did not alter the R3230AC tumor growth rate. Hypophysectomy decreased tumor growth (Hilf et al 1966b). Cohen and Hilf (1975) found that tumor growth was equal to or greater in diabetic rats than in intact animals.

Respirometric Studies

The R3230AC tumor resembled normal mammary tissue in its inability to utilize glucose under anaerobic conditions (Hilf et al 1965a). This was unusual because malignant tissues are generally capable of anaerobic metabolism.

Isoenzymes

Lactate dehydrogenase (LDH) isoenzymes and glucose 6-phosphate dehydrogenase (G6-PDH) isoenzymes were studied. (Richards and Hilf 1972 and Williams and Fritz 1973). Many investigators have shown that these enzymes, particularly the LDH-3, LDH-4 and LDH-5 isoenzymes, are elevated in neoplastic tissues. In the studies dealing with the R3230AC tumor the researchers found that the mammary gland of normal rats contained equal proportions of G6-PDH-1 and G6-PDH-2 plus considerable amounts of LDH-1 and LDH-2. In contrast, the tumor tissues contained higher total activities of G6-PDH and LDH. Glucose 6-phosphate dehydrogenase-2, LDH-4 and LDH-5 were the predominent isoenzymes.

Virus Particles

Chopra and Taylor (1970), utilizing the electron microscope, were unable to demonstrate the presence of virus particles in the R32030AC tumor.

The R3230AC Tumor as a Research Model

Most of the work using the R3230AC tumor was aimed directly or indirectly at finding the similarities and differences between the tumor and normal mammary tissue. The final goal was to develop a uniform hypothesis for the mechanism of neoplastic transformation. The conclusions of the these studies frequently proposed more questions than they answered, but the information gained provided better direction for further research.

Many of the studies using the R3230AC tumor provided insights into neoplastic phenomena in general. Hilf *et al* (1971) concluded that the

known principles of hormonal control of normal endocrine target organs may not necessarily be applicable to control of similar processes in endocrine responsive tumors. Through studies on hormonal effects Hilf et al (1965a, 1968) concluded that decreased tumor enzyme activities do not impair the ability of the neoplasm to grow. Costlow et al (1974) found the R3230AC tumor useful for studying the relationship between malignant transformation and the presence of hormone receptors on tumor cells. On the other hand, Harmon and Hilf (1976) concluded that neoplastic transformation does not necessarily alter the character of the insulin receptor. Shin et al (1975), through cell membrance studies, proposed that glycoprotein differences between normal and neoplastic tissues may be important in neoplastic behavior. Cohen and Hilf (1975) showed that the metabolic consequences of hormonal treatment in vivo may not always be duplicated in vitro.

The R3230AC tumor was used in a more specific way for insights into mammary neoplasia. McGuire (1969) used the R3230AC carcinoma as an experimental model of the mechanism of hormonal regulation of growth and differentiation of mammary carcinoma cells. Shafie et al (1977) used the R3230AC tumor in altered endocrine environments to obtain information regarding both the pathophysiology of mammary cancer and the regulation of hormone receptors. Smith et al (1977) used the tumor as an animal model for the elucidation of the growth and metabolic responses to prolactin in breast cancer. Along a similar line, Hilf et al (1967) was able to refute the theory that the stimulation or inhibition of mammary tumor growth was directly reflected by plasma prolactin levels in the tumor bearing animals. Through studying the R3230AC

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tumor, Turkington (1974) concluded that the degree of prolactin dependence of certain mammary carcinomas may be characterized by the relative numbers of prolactin receptors present. In a paper given at a symposium on cancer, Hilf (1970) summarized much of the research to date on the R3230AC tumor. Through comparisons with several other mammary tumors, including some of man, he showed that the R3230AC tumor was a valid and valuable animal model for human breast cancers, particularly those of the autonomous hormone-responsive type. Cohen and Hilf (1975) proposed more specificly that the R3230AC may represent an experimental model of breast cancers in women which recur after ablation of hormone-producing organs. The recurrent tumor may be different from the original lesion which may have been hormone dependent. Everson et al (1973) used the tumor as an animal model system for testing an antitumor agent. The results of the isoenzyme work of Richards and Hilf (1972) indicated that isoenzyme alterations may be used as a predictive measure of the efficacy of a therapeutic measure in the treatment of certain cancers.

Along a more general vein, Prasad et al (1979) found the R3230AC tumor to be a useful model for studying protein glycosylation and the biological control of the synethesis of α-lactalbumin.

Alanine Aminotransferase

Chemistry

The enzyme, alanine aminotransferase (ALT, glutamic pyruvic transaminase, GPT) is designated by Enzyme Commission number 2.6.1.2. It catalyzes the following reaction:

alanine + α - ketoglutarate $\xleftrightarrow{ALT}$ glutamate + pyruvate

Pyridoxal phosphate, vitamin B-6, is a cofactor in the reaction. The enzyme has a high degree of specificity for its substrates (Segal et al 1962). Its molecular weight is 114,000 (Gatehouse et al 1967, Braunstein 1973) and it exists as a dimer (Braunstein 1973).

Species Variation

There is a great deal of species variability in the organ distribution of the enzyme. In man, the enzyme was highest in the liver, skeletal muscle, heart, kidney and pancreas (Wroblewski and LaDue 1956, Kamoda et al 1980). In ruminants the highest levels were found in the heart and relatively low levels of alanine aminotransferase were found in the liver (Cornelius et al 1959, Boyd 1962, Baetz 1970). In swine alanine aminotransferase levels were highest in skeletal muscle, the brain and pancreas (Cornelius et al 1959). The liver, heart, kidney and pancreas contained relatively high levels of the enzyme in the horse (Cornelius et al 1959, Baetz 1970). In the dog and rat the highest activities were found in the liver (Awapara and Seale 1952, Boyd 1962).

Diagnostic Application

The value of alanine aminotransferase as a serum indicator of hepatocellular damage in man was first recognized in 1956 by Wroblewski and LaDue and by DeRitis et al. Cornelius et al (1959) advocated the assay of serum alanine aminotransferase as a diagnostic test for hepatic necrosis in the canine and Boyd (1962) found it useful in the rat. The enzyme was of no diagnostic value in ruminants, the horse, pig or chicken. In veterinary medicine it is presently in common usage as a clinical test for hepatic injury in the dog, the cat and primates (Coles 1974, Duncan and Prasse 1977 and Benjamin 1978). Rats with hepatic damage are reported to have a similar enzyme profile to that of the dog (Friedman and Lapan 1964). In the serum the alanine aminotransferase activity is found in the albumin, α_1, α_2 β and γ-globulin fractions. The distribution varies in different disease states (Demetriou et al 1974).

Location in Rat Tissues

In the rat, alanine aminotransferase values are essentially zero in all tissues except the liver (Boyd 1962, Knox 1976). In the rat liver the highest enzyme activity is in the portal region. The second highest activity is in the midzonal region and the lowest activity is in the centrilobular region (Shank et al 1959, Morrison et al 1965). Within the individual hepatocytes, there are two isoenzymes, a cytosolic ALT and a mitochondrial ALT (Kafer and Pollack 1961, Segal et al 1962, Swick et al 1965, Radhakrishnamurry and Sabry 1968, and Herzfeld et al 1976). The mitochondrial isoenzyme is the major form in fetal rat liver. It decreases to an insignificant amount soon after birth. In man, the particulate (mitochondrial) form persists after birth (Herzfeld et al

1976). The mitochondrial isoenzyme is highly labile with a half life of 6 hours. Efforts to purify this isoenzyme were not successful. In contrast, the cytosolic enzyme is highly stable in the crude or purified form for over two months when stored at 0°C (Swick et al 1965).

Factors Influencing Liver Alanine Aminotransferase Values

Alterations in the activity of ALT in the rat liver can be brought about by several factors. Fasting caused an increase in the enzyme activity (Rosen et al 1959, Soberón and Sanchez 1961 and Nakata et al 1964b). A protein deficient diet resulted in decreased liver ALT activity while an excess of protein in the diet caused increased liver ALT activity (Rosen et al 1959, Nakata et al 1964a). A vitamin B-6 deficient diet produced decreased activity of liver ALT, and an excess of the vitamin produced an increase in liver ALT activity (Brin and Thiele 1967 and Radhakrishnamurty and Sabry 1968). The glucocorticoids, cortisone, hydrocortisone and prednisolone caused an increase in liver ALT activity (Segal et al 1962, Nakata et al 1964a, and Isohashi et al 1980). Degenerating hepatocytes contained decreased ALT activity (Morrison et al 1965). Conflicting results were obtained regarding regenerating hepatocytes. Sanchez et al (1961) reported decreased liver ALT while Nakata et al (1964b) observed increased ALT. Diabetes resulted in increased liver ALT (Rosen et al 1959). Pregnancy had no effect on liver ALT (Nakata et al 1964a). Rats bearing the AH130 ascites tumor had increased liver ALT activity in the absence of any hepatic lesions (Nakata et al 1964a and Isohashi et al 1980).

Factors Influencing Serum Alanine Aminotransferase Values

Liver necrosis in the rat will produce a large increase in serum alanine aminotransferase (Boyd 1962). In some instances the elevated serum level may be associated with a decrease in the level of the enzyme in the tissue due to a loss from the tissue into the serum (Boyd 1962). With the necrosis of hepatocytes cytosolic enzymes continue to efflux until equilibrium with the environment is achieved (Schmidt, F. W. 1978). Frank necrosis is not required for the serum elevation of ALT. Because the enzyme is cytosolic all that is necessary for its appearance in the serum is an alteration in cell membrane permeability (Benjamin 1978 and Schmidt, E. 1978). This can be due to a perturbation in the cell causing biochemical alterations without any histologic evidence of damage. Friedrich W. Schmidt (1978) demonstrated elevated levels of several serum enzymes, including ALT, in the absence of histologic lesions. He concluded that the release of cellular enzymes is one of the most sensitive indexes for the recognition of minute disturbances of cellular integrity. The exact mechanism of this release is unknown however. It may also happen that there is an increase in synthesis of the enzyme in the liver so that a concomitant drop in the tissue level is not seen with elevation in the serum (Boyd 1962, Friedman and Lapan 1964, Brin and Thiele 1967).

Elimination from the Body

Once released from the cell the enzyme is rapidly distributed throughout the body fluids. The site of elimination of ALT from the body is unknown (Fleisher and Wakim 1963, Schmidt, F. W. 1978). There may be a gradual loss of activity in the body, or the enzyme may be metabolized, or it may be excreted partially metabolized.

MATERIALS AND METHODS

Transplantation

Donor rats were killed at 5 weeks post-transplantation using carbon dioxide vapor from dry ice.[a] The lungs were immediately exposed via a ventral midline incision. Metastatic nodules measuring 2mm diameter or less were removed and placed in cold sterile physiologic saline which was maintained in an ice bath. The nodules were minced with two scalpel blades. A piece of the nodule approximating $2mm^3$ was placed in the bevel of a 19 gauge one inch needle[c] which was then used as a cannula for insertion of the tumor into the recipient rat. A 22 gauge 1 1/2 inch needle with its bevel ground flat was inserted into the 19 gauge needle and was used to push the tumor piece through once the larger needle's bevel was beneath the renal capsule in the recipient rat (Figure 2).

Recipient rats were weanling female Fischer rats[b] weighing between 60 and 90 grams. For ease of handling, the rats were first sedated with carbon dioxide vapor from dry ice and then immediately anesthesized with an intraperitoneal injection of sodium pentobarbital

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[a]Cardox®, Division of Chemetron Corporation, Countryside, Illinois

[b]Fischer 344/NUpj, The Upjohn Company, Kalamazoo, Michigan

[c]Yale Hypodermic Needle, Becton, Dickinson and Company, Rutherford, New Jersey

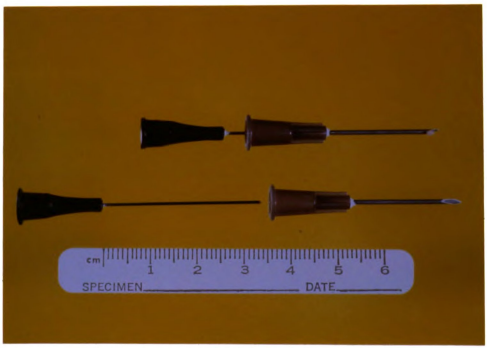

Figure 2. Nineteen and 22 gauge needles used for tumor implantation. The bevel of the 22 gauge needle has been filed so that the tip is closed and flat. The bevel of the upper 19 gauge needle contains a piece of metastatic tumor of the size ideal for transplantation.

at a dosage of 0.03 mg/gm body weight. The dorsal lumbar region was clipped with an electric clipper containing a #40 blade. The surgical site was cleaned and disinfected.[d] The paralumbar surgical approach was used to expose the right kidney and a piece of harvested metastatic nodule was inserted under the renal capsule using the 19 and 22 gauge needles as cannula and trochar. Rats, which served as sham matched controls, were handled similarly except that the trochar and cannula inserted beneath the kidney capsule were without a tumor piece.

The R3230AC-L line has been maintained since 1973 by repeated transplants using the above method. Each successive transplant generation was given a L-number which indicated the number of transplants generations that have occurred since the tumor line began. For example, the L-55 designation indicated that the tumor line was into the 55th transplant generation in the lung.

Hematology and Clinical Chemistry

Blood samples were taken from the posterior vena cava and placed in vials[e] containing ethylenediamine tetraacetic acid. These samples were used for the determination of the following:

1. Hemoglobin content (Hb)
2. Hematocrit (HCT)
3. Total erythrocyte count
4. Total leukocyte count

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[d]Mercresin, The Upjohn Company, Kalamazoo, Michigan

[e]Vacutainer, Becton, Dickinson and Company, Rutherford, New Jersey

5. Mean corpuscular volume (MCV)
6. Mean corpuscular hemoglobin (MCH)
7. Mean corpuscular hemoglobin concentration (MCHC)
8. Differential leukocyte count.

The hemoglobin content, packed cell volume, total erythrocyte count, total leukocyte count and erythrocytic indices were determined with an automated electronic analyzer.[f] The differential leukocyte count was derived by counting 100 white blood cells in a blood smear stained with Wright's stain.[g]

For clinical chemistry determinations blood samples were placed in silicone coated glass vials[h] and allowed to clot. The serum was aspirated from the clot after centrifugation at 2000 revolutions per minute for 15 minutes. A centrifical analyzer[i] was used to determine the serum levels of the following chemical constituents:

1. Alanine aminotransferase (Wróblewski and LaDue 1956)
2. Aspartate aminotransferase (Karmen 1955)
3. Sorbitol dehydrogenase (Sigma Technical Bulletin 1972)
4. Alkaline phosphatase (Bessey et al (1946)
5. Gamma glutamyl transpeptidase (Szasz 1969)
6. Leucine aminopeptidase (Szasz 1967)
7. Lactate dehydrogenase (Amador et al 1963 and Gay et al 1968)
8. Total bilirubin (Centrifichem 1980)

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[f]Coulter S., Coulter Electronics, Inc., Hialeah, Florida

[g]Camco Quick Stain, Cambridge Chemical Products, Fort Lauderdale, Florida

[h]Vacutainer, Becton, Dickinson and Company, Rutherford, New Jersey

[i]Centrifichem, Union Carbide Corp., Rye, New York

9. Direct bilirubin (Centrifichem 1980)

10. Total protein (Weichselbaum 1946)

11. Albumin (Rodrey 1964 and Dow and Pinto 1969)

12. Blood urea nitrogen (Centrifichem 1980)

Homogenization of Tissue Samples

The rat was weighed immediately prior to euthanasia with carbon dioxide vapor. Tissues for enzyme determinations were placed in 10 volumes of cold 0.15 molar saline as quickly as possible after removal and after weighing on an electric analytical balance.[j] Representative samples were thinly cut and were placed in a fresh container of cold 0.15 molar saline. This was done in order to remove as much blood from the tissue as possible. A 100 mg tissue sample was then placed in a volumetric flask with sufficient 0.15 molar saline to bring the total volume to 10 ml.[k] The samples were maintained in an ice bath at all times to prevent enzymatic degredation. The contents of the volumetric flask were then transferred to a 10 ml Potter-Elvehjem glass homogenizer[l] fitted with a motor driven[m] teflon pestle. Each sample was homogenized in an ice bath for 2 one-minute periods at 1000 revolutions per minute according to the method of Schneider (1961).

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[j]Mettler P16Q, Mettler Instrument Corporation, Hightstown, New Jersey

[k]Appendix 1. Media, Dialysis and Storage Effects upon Alanine Aminotransferase and Hemoglobin in Liver and Kidney Homogenates

[l]A. H. Thomas Company, Philadelphia, Pennsylvania

[m]Tri-R Stir-R Model K41, Tri-R Laboratory Instruments, Inc., New York, New York

The samples were then transferred to centrifuge tubes and were spun in a pre-cooled refrigerated centrifuge (2-4°C)[n] for 15 minutes at 1500 x g. The supernatant was decanted and then centrifuged again in a high speed refrigerated centrifuge (2-4°C)[o] at 39,300 x g for one hour. The supernatant was decanted into glass containers which were sealed and stored in a refrigerator at 5-15°C until enzymatic analysis could be performed the next day.

The homogenate sample, the serum sample and a control serum sample[p] were analyzed for alanine aminotransferase and for hemoglobin using a centrifical analyzer.[q] The control serum served to assure consistency of results. The method for the analysis for alanine aminotransferase was according to Wrobewski and LaDue (1956). The method for the analysis for hemoglobin was according to Standefer and Vanderjagt (1977). The activity of alanine aminotransferase in the tissues was calculated on a per gram of tissue (wet weight) basis. The hemoglobin values were used to factor out that part of the tissue alanine amino-transferase which was due to blood contamination according to the formula: Corrected tissue alanine aminotransferase = measured tissue alanine aminotransferase x $(1 - \frac{\text{tissue hemoglobin}}{\text{blood hemoglobin}})$.

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[n]Beckman, Model TJ-6, Beckman Instruments, Inc., Palo Alto, California

[o]Sorvall Superspeed, Model RC2-B, Dupont Instruments, Biomedical Division, Newtown, Connecticut

[p]Moni-Trol I, Dade Division American Hospital Supply Corporation, Miami, Florida

[q]Centrifichem, Union Carbide Corporation, Rye, New York

Post Mortem Examination

The rats were placed in a bell jar containing dry ice to euthanize them by an excess of carbon dioxide vapor. According to Feldman and Gupta (1976), this method of euthanasia produced no histologic changes in the rat. A complete post mortem examination consisted of careful observation of all thoracic and abdominal viscera and of the brain. All lesions and selected tissues were placed in 10 volumes of 10% neutral buffered formalin. After fixation for a minimum of 48 hours the tissues were trimmed and paraffin[r] embedded with an automated tissue processor.[s] They were then sectioned at a thickness of 5 microns and stained with hemotoxylin, eosin and phloxine. All staining was according to the methods outlined in The Pathology and Toxicology Unit's Manual of Standard Operating Procedures (1979).

Electron Microscopy Procedures

Specimens for ultrastructural examination were collected immediately after euthanasia and were placed in phosphate buffered 2% gluteraldehyde. They were processed according to Luft (1961) using osmic acid fixation, graded concentrations of alcohol for dehydration and epon for embedding. Thick sections were stained with toluidine blue. Thin sections were stained with uranyl magnesium acetate and

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[r]Paraplast, Lancer Division of Sherwood Medical Industries, St. Louis, Missouri

[s]Autotechnicon, Technicon Company, Tarrytown, New York

lead citrate. The samples were examined and electron photomicrographs were taken with a transmission electron microscope.[t]

Electrophoresis

Liver, tumor and metastatic tumor tissues were homogenized as outlined previously with the exception that the amount of tissue used was 200 mg instead of 100 mg. Once the homogenization procedure was completed, the samples were refrigerated in sealed containers at 10°C if the electrophoresis was to be done within 24 hours. If a longer storage time was required, the samples were frozen at -60°C and then defrosted at room temperature just prior to electrophoresis.

The electrophoretic procedure was a modification of the method of Chen and Giblett (1971). The starch gel was prepared 1 day in advance using 9.5 grams of hydrolyzed starch,[u] 10 ml of a 0.1 molar solution of Trizma base[u] and citrate, and 90 ml distilled water buffered to a pH of 7.5. The electrophoretic buffer tanks[v] contained a 0.1 molar solution of Trizma base and citrate buffered to a pH of 7.5. The homogenate samples were pipetted into wells cut in the starch gel. The plate was placed on a horizontal cooling plate[w] precooled to 10°C. The wells were located on the cathode side. The enzyme migrated to the anode. The power source[x] was set to run at either 2.75 volts/cm for 16 hours or at 8 volts/cm for 6 hours.

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[t]Philips 201, Philips Corporation, Eindhaven, Netherlands

[u]Sigma Chemical Company, St. Louis, Missouri

[v]Multiphor, LKB, Rockville, Maryland

[w]Multi Temp, LKB, Rockville, Maryland

[x]LKB 2103, LKB, Rockville, Maryland

The gel was stained according to the method of Chen and Giblett (1971). This method uses the same reagents as if the sample were being analyzed using a UV spectrophotometer. One vial of premeasured reagents[y] dissolved with 10 ml distilled water provided approximately the same concentration of reagents as reported by Chen and Giblett.[z] A piece of Whatman No. 1 filter paper was placed over the gel and then saturated with the contents of the reagent vial. The plate was warmed to 24°C and the staining reaction was allowed to continue for 3 hours. The filter paper was removed at the end of 3 hours and the gel was examined with a long wave ultraviolet light. The areas of negative fluorescence were the sites of the alanine aminotransferase migration. The gel was photographed under the ultraviolet light using a Polaroid camera[aa] and a #58 Wratten filter.[bb]

Statistical Analysis

The parametric data were analyzed using the analysis of variance completely random design (Steel and Torrie 1960). Significance was determined at the 0.05 level of probability.

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[y]GPT 10 Bio-Dynamics/Gmc, Indianapolis, Indiana

[z]Appendix 2. Reagents for Staining ALT Electrophoresis

[aa]Polaroid Corporation, Cambridge, Massachusetts.

[bb]Eastman Kodak Company, Rochester, New York.

EXPERIMENTAL DESIGN

Study I: Life Expectancy and Time-Course Study

Objective: This study was designed to determine the life expectancy of the tumor-bearing rats and to follow the alterations with time of hematologic values and serum enzymes.

1. Animals. A total of 89 female Fischer 344 rats were used. The rats were weighed. Sixty were given the R3230AC-L tumor and 29 were sham operated as previously described. This was the 55th transplant generation of the L line. The rats were observed twice daily for general health and palpated weekly to assess tumor growth.
2. At 2 week intervals following the surgery 10 tumored and 5 control rats were randomly selected and killed. The following observations were recorded and samples were collected for further evaluation:
    1) Body weight
    2) Blood samples for:
        (a) Complete blood count - hemoglobin content, hematocrit, total erythrocyte count, total leukocyte count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, differential leukocyte count.
        (b) Serum clinical chemistries - alanine aminotransferase, aspartate aminotransferase, sorbitol dehydrogenase, alkaline phosphaste, gamma glutamyl transpeptidase

leucine aminopeptidase, lactate dehydrogenase, total bilirubin, direct bilirubin, total protein, serum albumin, blood urea nitrogen.

3) A complete post mortem examination.

4) The dimensions of the renal tumor.

5) The number of grossly visible lung metastases.

6) Organ and tissue weights:

(a) Lung - including metastatic nodules.

(b) Liver

(c) Left kidney

(d) Right kidney - tumor removed

(e) Tumor - separated from the kidney

4) Samples collected for histopathologic examination:

(a) Brain

(b) Heart

(c) Left kidney

(d) Right kidney

(e) Liver

(f) Lung

(g) Spleen

(h) Tumor

(i) Any gross lesion(s)

3. The remaining rats were allowed to live until severe clinical signs developed. These were then euthanatized and necropsied. Only histologic specimens were taken from those rats found dead in the cage or those killed because of a moribund condition.

Study II: Enzyme Activity in Liver and Tumor Homogenates

Objective: The purpose of this study was to determine the source of the elevated serum alanine aminotransferase.

1. Animals. Female Fischer 344 rats were used at 6 weeks post-transplantation. No more than 5 tumor-bearing rats and 5 control rats were processed on one day due to the amount of time needed to prepare the tissues.
2. Three generations of tumor were tested:
    1) L-58 - 4 tumor-bearing rats and 4 control rats
    2) L-59 - 10 tumor-bearing rats and 10 control rats
    3) L-60 - 10 tumor-bearing rats and 10 control rats
3. The following observations were recorded and samples were collected for further evaluation:
    1) Body weight
    2) Blood samples for:
        (a) Hemoglobin
        (b) Serum alanine aminotransferase
    3) Organ and tissue weights:
        (a) Liver
        (b) Primary tumor - separated from the kidney
        (c) Lung - including metastatic nodules
    4) Tissues for homogenization, light microscopy and electron microscopy:
        (a) Liver
        (b) Primary tumor - separate homogenates were processed from the peripheral, more viable tumor tissue and from the central necrotic tissue

5) Homogenate samples were analyzed for:

(a) Hemoglobin

(b) Alanine aminotransferase

Study III: Electrophoresis of Serum and Liver and Tumor Homogenates

Objective: This study was designed to compare the alanine amino-transferase derived from the different tissues of control and of the R3230AC-L tumor-bearing rats.

1. Animals. Female Fischer 344 rats were used at 5 to 6 weeks post-transplantation.
2. Groups. Each group consisted of 3 rats, 2 tumor-bearing and 1 control. Three tumor generations were sampled.
    1) L-66 - Group I
    2) L-66 - Group II
    3) L-67 - Group III
    4) L-67 - Group IV
    5) L-68 - Group V
3. The following observations were recorded and samples were collected for further evaluation:
    1) Body weight
    2) Blood samples for:
        (a) Hemoglobin
        (b) Serum alanine aminotransferase
    3) Organ and tissue weights:
        (a) Liver
        (b) Primary tumor - separated form the kidney

(c) Lung with metastatic nodules

4) Homogenate samples:

   (a) Liver

   (b) Primary tumor

   (c) Metastatic nodules

5) Electrophoretic samples:

   (a) Serum

   (b) Liver homogenate

   (c) Primary tumor homogenate

   (d) Metastatic nodule homogenate

6) The excesses of the samples for electrophoresis were stored frozen at -60°C. These were later thawed and retested for hemoglobin and alanine aminotransferase and re-electrophoresed to determine if freezing produced any change.

RESULTS

Study I: Life Expectancy

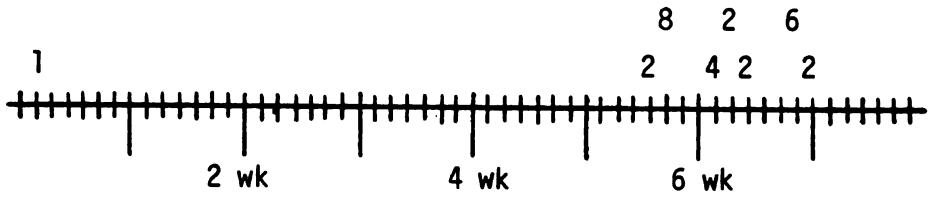
The lifespan of rats bearing the R3230AC adenocarcinoma is illustrated in Figure 3. One rat died on the first day after surgery as a result of surgical complications. Most of the rats died near 6 weeks post-transplantation. In this study no rat survived beyond seven weeks. Clinical signs just prior to death included severe dyspnea and cachexia. Post mortem examination revealed a mottled, slightly nodular neoplasm approximately 3 by 2 cm replacing the right kidney, nearly complete metastatic infiltration of the lungs and an absence of internal body fat. Implanted renal tumors generally did not infiltrate beyond the right kidney and did not appear to be large enough to have been detrimental to the abdominal viscera or cause the death of the animal.

Study I: Time-Course Changes

Gross Pathologic Observations

Post mortem examination of the rats at 2 weeks after implantation revealed the presence of the tumor in the right kidney (Figure 4). At this stage of development, tumors could be separated from renal tissue with relative ease. The mean tumor weight was 0.27 $\pm$ 0.03 gm.[a] The

[a] Mean value $\pm$ standard deviation of the mean.



Tumor Wt. (g)	.27	2.47	5.14
Metastases	0	46	TNTC
Metastases' diameter (mm)	0	0.5-3.0	0.5-6.0
ALT (IU/L)	15	73	208

Figure 3. The numbers above the time line indicate the number of tumor-bearing rats which died in addition to the scheduled biweekly groups. Also shown are the mean tumor weights, metastases numbers and diameters and mean serum alanine aminotransferase activity for the biweekly killed groups of tumor-bearing rats.

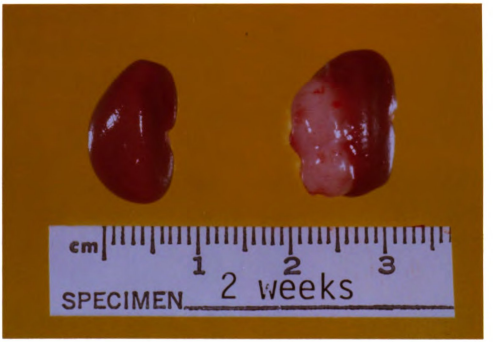


Figure 4. Control kidney on the left and kidney with the R3230AC-L tumor 2 weeks after implantation.

4 week post-transplantation group had a mean tumor weight of 2.47 $\pm$ 1.33 gm (Figure 5) and there were metastatic nodules in the lungs (Figure 6). These varied in number from 5 to 75 and in diameter from 0.5 to 3 mm. The 6 week group had a mean tumor weight of 5.14 $\pm$ 1.45 gm (Figure 7) and the metastatic nodules in the lung at this stage were too numerous to count accurately (Figure 8). The diameters of these metastatic nodules ranged from 0.5 to 6 mm.

Histopathologic Observations

Histologic lesions in the 2 week group were confined to the right kidney and opposing liver tissue which had become adhered. Transplanted tumors grew into the kidney causing compression and progressive necrosis of the renal parenchyma (Figure 9). Hepatic tissue in direct contact with the right kidney, was invaded by the tumor in 3 of ten rats. A fourth rat had hepatic fibrosis at the point of contact with the tumor but there was no invasion of tumor cells into the liver.

Morphologically, tumors were characterized by relatively solid masses of epithelial type cells interspersed with a fine connective tissue stroma. The cells appeared quite anaplastic but were arranged in clusters resembling glandular acini. The cytoplasm varied from compact and finely granular to extremely expanded and foamy. Nuclei were pleomorphic, vesicular and frequently very large. Occasional very large nucleoli were observed. Mitotic figures, often atypical, were numerous (Figure 10). There were varying degrees of coagulative necrosis in the more central regions of the tumor. The tumor appeared to be growing by invasion as there was no distinct line between tumor cells and renal parenchyma and occassional glomeruli were oberved surrounded by

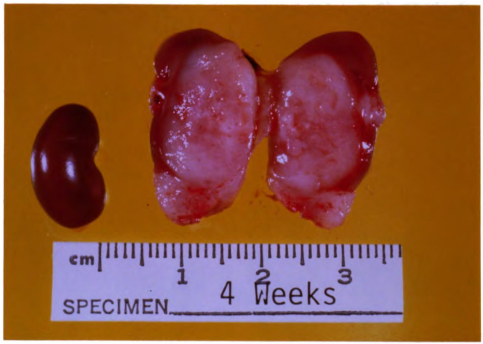


Figure 5. Control kidney on the left and a kidney with the R3230AC-L tumor 4 weeks after implantation. The kidney has been bisected to show the depth of penetration of the tumor.

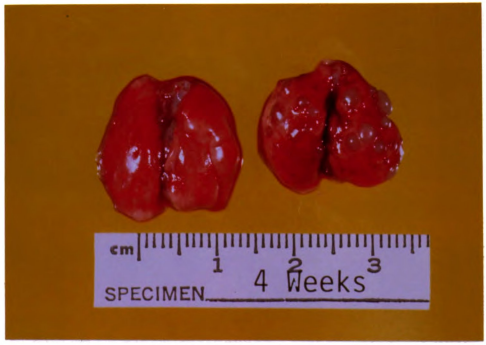


Figure 6. Lung from a control rat on the left and lung containing neoplastic nodules, the result of metastasis from the intrarenal implantation of the R3230AC-L adenocarcinoma 4 weeks previously.

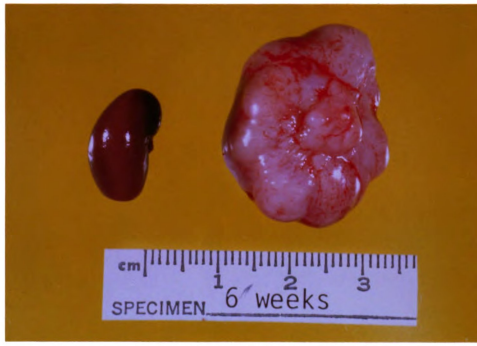


Figure 7. Control kidney on the left and a kidney with the R3230AC-L tumor 6 weeks after implantation. No renal tissue is grossly discernably from the neoplasm at this stage of tumor growth.

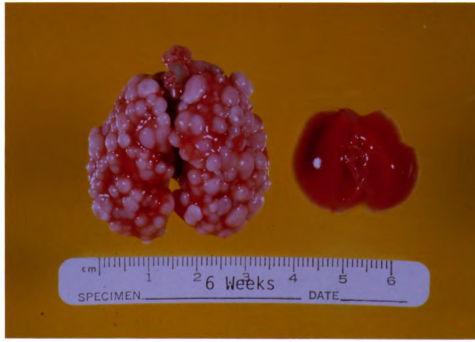


Figure 8. Control lung on the right and a lung greatly enlarged by the presence of innumerable metastatic nodules from the intrarenal implantation of the R3230AC-L tumor 6 weeks previously.

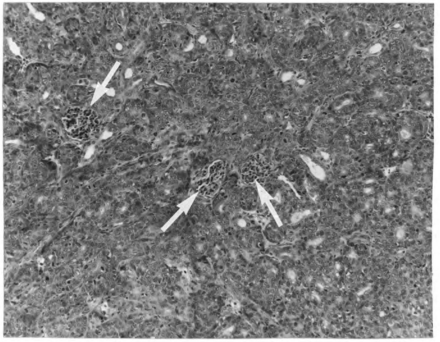


Figure 9. The R3230AC-L tumor 2 weeks after implantation. Notice the densely cellular appearance and glomeruli (arrows) surrounded by neoplastic cells. H&E stain, 100X.

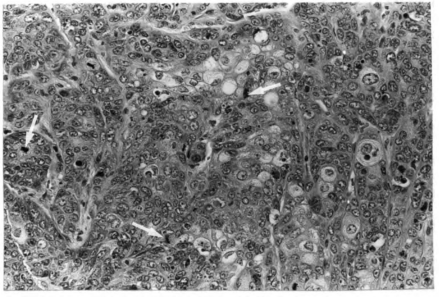


Figure 10. The R3230AC-L tumor 2 weeks after implantation. Notice the large foamy cells, numerous mitotic figures (arrows) and overall anaplastic appearance. H&E stain, 640X.

neoplastic cells. In the right kidneys of control rats, there was frequently a line of macrophages and slight tubular degeneration extending from a small depression at the cortical surface into the parenchyma.

In the 4 week post-transplantation group histologic lesions were observed in the lungs, right kidney and right lateral lobe of the liver. In 2 of 10 rats there was infiltration of the tumor into the liver at the site of apposition. There were larger areas of necrosis with replacement fibrosis occurring in some of the necrotic tissue (Figures 11 and 12). The overall histologic appearance of the tumor was essentially the same as the 2 week group (Figure 13). Necrosis of the right kidney was more apparent due to further invasion of the growing tumor. The lung contained variable sized round foci of metastatic tumor cells (Figure 14). These were scattered throughout the pulmonary parenchyma with no apparent predilection for any particular region. Cells in the bigger nodules were larger due to the abundance of foamy cytoplasm suggestive of secretory activity (Figure 15). In addition, some of the larger nodules contained focal areas of caseation necrosis loacted in the more central regions. Two of 5 control animals had evidence of a linear subacute to chronic inflammatory reaction in the right kidney extending from the cortical surface into the parenchyma.

The 6 week post-transplantation group had lesions in the lung, right kidney and liver. The lung was almost completely obliterated by the increased numbers and size of metastatic nodules (Figures 16 and 17). Coagulative necrosis was prominent in the larger nodules. At this stage of development there was only a thin remnant of the right kidney remaining and this contained infiltrating malignant adenocarcinoma

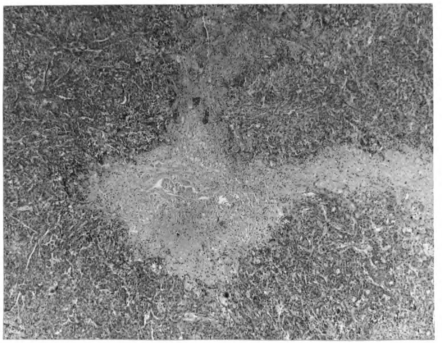


Figure 11. The R3230AC-L tumor 4 weeks after renal implantation. Note the large area of necrosis. H&E stain, 100X.

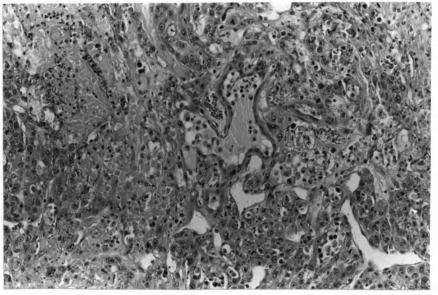


Figure 12. The R3230AC-L tumor 4 weeks after renal implantation. Notice the coagulative necrosis of neoplastic cells. H&E stain, 400X.

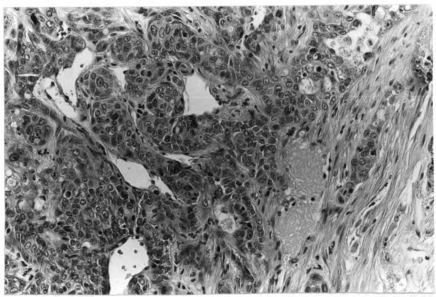


Figure 13. Cellular detail of the R3230AC-L tumor 4 weeks after intrarenal implantation. Note the lack of secretory activity and replacement fibrosis of the necrotic areas. H&E stain, 500X.

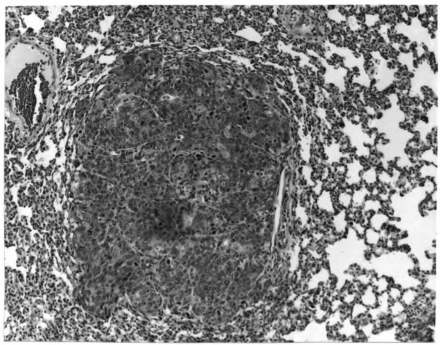


Figure 14. Typical appearance of a metastatic nodule in the lung 4 weeks after intrarenal implantation of the R3230AC-L adenocarcinoma. H&E stain, 100X.

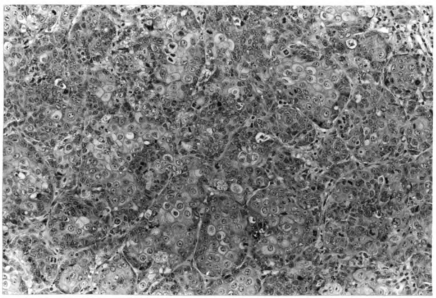


Figure 15. Cellular detail of the metastatic tumor 4 weeks after intrarenal implantation of the R323OAC-L tumor. Notice the greater number of foamy, secretory type cells compared to the primary tumor. H&E stain, 400X.

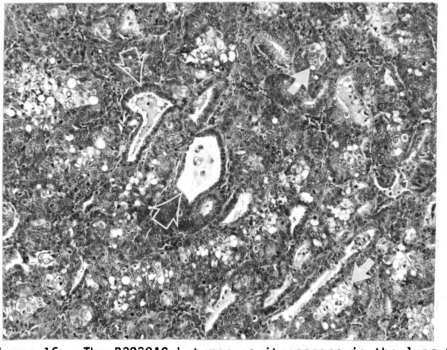


Figure 16. The R3230AC-L tumor as it appears in the lung 6 weeks after intrarenal implantation. Notice the ductile structures (open arrows) and large foamy secretory cells (solid arrows). H&E stain, 100X.

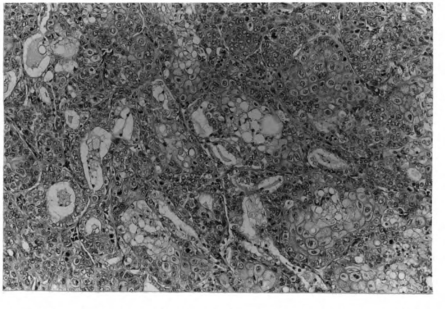


Figure 17. Cellular detail of the R3230AC-L tumor metastatic to the lung 6 weeks after intrarenal implantation. H&E stain, 400X.

cells. Necrosis was more extensive than in the previous two groups and frequently the only viable tumor tissue was that adjacent to blood vessels (Figures 18 and 19). Five rats had infiltration of the tumor cells across the adhesions and into the liver along the hepatic border which was in direct contact with the implanted renal tumor. Another rat had an area of fibrosis, necrosis and hemorrhage at the tip of the liver where it had become adhered to the right kidney. Two control rats in the 6 week group had fibroblastic connective tissue at the cortical surface of the right kidney where adhesions had formed between the liver and kidney. One control rat had a small indentation of the cortex of the right kidney with a line of mononuclear inflammatory cells extending into the cortex. The left kidney also had a small cortical indentation with a small focus of lymphocytes beneath the depression.

Body and Organ Weights

Organ weights were statistically adjusted for the differences in total body weights and the means were compared to control means (Table 1). In the 2 week group significant differences ($P<0.05$) were found in terminal body weights and weights of the right kidney. Among the 4 week group, significance was found only in the right kidney weights. In the 6 week group there were significant differences in terminal body weights, lung weights, left kidney weights and right kidney weights.

Hematology

Statistically significant differences ($P<0.05$) in the 2 week group were seen in total erythrocyte count and mean corpuscular hemoglobin (Table 2).

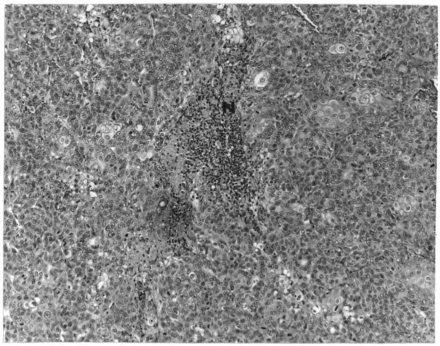


Figure 18. The R3230AC-L tumor 6 weeks after implantation. The stippled area (N) is an area of necrosis infiltrated with large numbers of neutrophils. H&E stain, 100X.

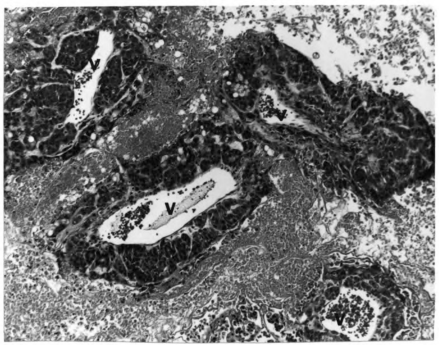


Figure 19. The R3230AC-L tumor 6 weeks after implantation. There is extensive coagulative necrosis and the only viable tissue is that surrounding blood vessels (v). H&E stain, 100X.

Table 1. Mean body and organ weights[a] (grams) expressed as mean ± standard error of the mean. N=10 for tumored rats and N=5 for control rats.

		2 Weeks	4 Weeks	6 Weeks
Initial Body Wt.	Tumored	78.4 ± 4.2	73.5 ± 4.0	82.7 ± 3.9
	Control	73.0 ± 5.9	79.6 ± 5.7	69.6 ± 5.5
Terminal Body Wt.	Tumored	111.6 ± 1.7[b]	132.2 ± 3.2	121.6 ± 5.4[b]
	Control	104.0 ± 2.4	131.1 ± 4.6	149.3 ± 7.9
Lung	Tumored	0.58 ± 0.02	0.86 ± 0.03	5.68 ± 0.58[b]
	Control	0.64 ± 0.02	0.79 ± 0.05	1.44 ± 0.87
Liver	Tumored	4.50 ± 0.12	6.04 ± 0.16	5.51 ± 0.14
	Control	4.59 ± 0.19	5.84 ± 0.23	5.27 ± 0.22
Left Kidney	Tumored	0.54 ± 0.01	0.74 ± 0.02	0.71 ± 0.01[b]
	Control	0.58 ± 0.02	0.68 ± 0.04	0.63 ± 0.02
Right Kidney	Tumored	0.49 ± 0.01[b]	0.55 ± 0.02[b]	0.42 ± 0.02[b]
	Control	0.55 ± 0.02	0.66 ± 0.03	0.63 ± 0.01

[a]Organ weights have been statistically adjusted for differences in individual body weights.

[b]Statistical significance with ANOVA ($p<0.05$).

Table 2. Mean hematologic values which showed statistical significance expressed as the mean ± standard error of the mean. N=10 for tumored rats and N=5 for control rats.

		2 Weeks	4 Weeks	6 Weeks
RBC($x10^6$/µl)	Tumored	7.33 ± 0.24[a]	7.33 ± 1.00	7.91 ± 0.30
	Control	7.85 ± 0.25	8.28 ± 0.36	8.11 ± 0.33
PCV(%)	Tumored	47.77 ± 1.18	43.21 ± 5.00	45.24 ± 1.97
	Control	49.10 ± 0.66	48.56 ± 1.12	47.48 ± 3.15
MCH(pg/red cell)	Tumored	20.31 ± 0.42[a]	19.29 ± 0.37	19.28 ± 0.58
	Control	19.57 ± 0.12	19.10 ± 0.46	19.28 ± 0.29
WBC($x10^3$/µl)	Tumored	6.60 ± 2.37	9.04 ± 1.89	9.83 ± 1.20[a]
	Control	4.23 ± 1.55	6.76 ± 0.58	6.46 ± 1.38
Seg. Neutrophils(%)	Tumored	13.0 ± 6.2	25.4 ± 7.4[ba]	52.5 ± 21.1[a]
	Control	12.7 ± 5.5	14.2 ± 4.6	16.0 ± 7.0
Lymphocytes(%)	Tumored	84.7 ± 5.7	73.4 ± 7.0[a]	45.6 ± 21.0[a]
	Control	87.0 ± 6.0	84.2 ± 5.6	82.8 ± 7.9

[a]Statistical significance with ANOVA ($p<0.05$).

In the 4 week post-transplantation group, statistically significant differences were seen in the total leukocyte count, packed cell volume, segmented neutrophil count and lymphocyte count.

In the 6 week post-transplantation group, statistically significant differences were seen in the total leukocyte count, segmented neutrophil count and lymphocyte count.

Serum Clinical Chemistries

Tests which had a statistically significant difference from control values in one or more groups are shown in Table 3. There was an increase in lactate dehydrogenase values in tumored rats of the 2 week group. In the 4 week post-transplantation group there was an increase in alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transpeptidase and lactate dehydrogenase and a decrease in alkaline phosphatase. Similar changes were seen in the 6 week post-transplantation group in addition to an increase in leucine aminopeptidase in the tumor recipients. No statistical differences were detected in the following tests: sorbital dehydrogenase, total and direct bilirubin, total protein, albumin and blood urea nitrogen.

Study II: Enzyme Activity in Liver and Tumor Homogenates

Alanine aminotransferase levels in serum and tissues from tumored rats are summarized in Table 4. The percent of total body weight contributed by each of the tissues is summarized in Table 5. From these data it can be seen that the tumor tissues (both primary and metastatic) did contain alanine aminotransferase. It can also be seen that they accounted for approximately 12% of the total body weight.

Table 3. Mean clinical chemistry tests (international units per liter) which showed statistical significance and expressed as the mean + standard error of the mean. N=10 for tumored rats and N=5 for control rats.

		2 Weeks	4 Weeks	6 Weeks
Alanine Aminotransferase	Tumored	14.6 ± 0.82	72.8 ± 6.44[a]	208.0 ± 25.32[a]
	Control	14.0 ± 1.16	16.0 ± 9.10	14.0 ± 35.81
Aspartate Aminotransferase	Tumored	76.2 ± 5.55	83.6 ± 4.67[a]	190.3 ± 21.77[a]
	Control	67.4 ± 7.85	49.0 ± 6.60	56.6 ± 30.79
Alkaline Phosphatase	Tumored	316.1 ± 11.19	232.5 ± 10.34[a]	114.2 ± 12.56[a]
	Control	350.4 ± 15.82	287.2 ± 14.62	235.2 ± 17.76
Gamma Glutamyl	Tumored	7.0 ± 0.84	7.2 ± 0.71[a]	8.0 ± 0.72[a]
Transpeptidase	Control	6.8 ± 1.19	2.4 ± 1.00	4.0 ± 1.02
Lactate Dehydrogenase	Tumored	448.7 ± 49.00[a]	262.1 ± 32.98[a]	520.2 ± 36.31[a]
	Control	195.6 ± 65.75	90.8 ± 46.63	295.8 ± 51.35
Leucine Aminopeptidase	Tumored	- -	25.4 ± 0.82	24.3 ± 0.58[a]
	Control	- -	25.6 ± 1.16	22.0 ± 0.82

[a]Statistical significance with ANOVA ($p<0.05$).

Table 4. Alanine aminotransferase activity in serum and tissues expressed as the mean ± standard error of the mean.

	Tumored N=24	Controls N=24
Serum (IU/l)	150.1 ± 11.1	18.8 ± 1.7
Liver (IU/gm)	22.5 ± 1.9	21.8 ± 0.5
Tumor - Peripheral (IU/gm)	4.3 ± 0.6	
Tumor - Central (IU/gm)	5.8 ± 1.0	
Metastases (IU/gm)	7.2 ± 0.1	

Table 5. Tissue percentage of total body weight

	Tumored N=24	Controls N=24
Liver	4.2 ± 0.1	3.6 ± 0.04
Tumor	6.9 ± 0.4	
Metastases[a]	4.8 ± 0.5	

[a]The little remaining normal lung tissue could not be separated from the metastatic nodules so this value included metastases and lung.

Gross and histologic observations were similar to those described in the previous study.

Electron Microscopy

Ultrastructural differences between tumored rat livers and control rat livers were not detected. Ultrastructural characteristics of renal tumors and pulmonary metastases were typical of adenocarcinomas as evidenced by microvilli and tight junctions. Characteristics of malignancy included the presence of prominent nucleoli and irregular nuclear membranes. Metastatic tumor cells appeared to have greater quantities of Golgi apparatus, rough endoplasmic reticulum and secretory products when compared with ultrastructural characteristics of the primary tumor (Figures 20 and 21).

Study III: Electophoresis of Serum and Liver and Tumor Homogenates

With regard to tumor bearing rats, there was no apparent difference on electrophorectic mobilities of alanine aminotransferase from serum and from homogenates of hepatic, primary tumor and metastatic tumor tissues. When these mobilities were compared to the mobilities of alanine aminotransferase from serum and hepatic tissue homogenates of control rats, no differences were observed (Figure 22).

ALT isoenzymes were not detected in either fresh or frozen serum and tissue homogenates from tumored and control rats. No differences were observed between samples subjected to eletrophoresis immediately after homogenization and those same samples subjected to electrophoresis after storage at -60°C.

Figure 20. Untrastructural detail of the primary R3230AC-L tumor. Typical of an adenocarcinoma is the presence of microvilli (MV) and tight junctions (arrows). Collagen fibrils (CF) are replacing necrotic areas. Glutaraldehyde - osmic acid fixation, uranyl magnesium acetate - lead citrate stain, 13,500X.

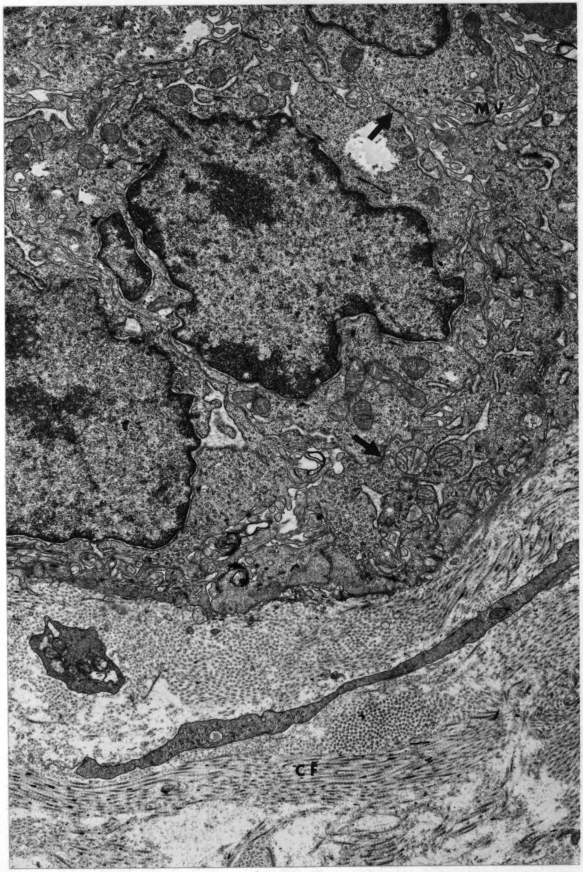


Figure 20

Figure 21. Utrastructural detail of the metastatic R3230AC-L tumor. Note the increased amount of secretory products (S) and dilated rough endoplasmic reticulum compared to the primary tumor. Microvilli (MV) and tight junctions (arrows) are also present. There are 2 structures suggestive of myelin figures (M). Glutaraldehyde - osmic acid fixation, uranly magnesium acetate - lead citrate stain, 13,500X.

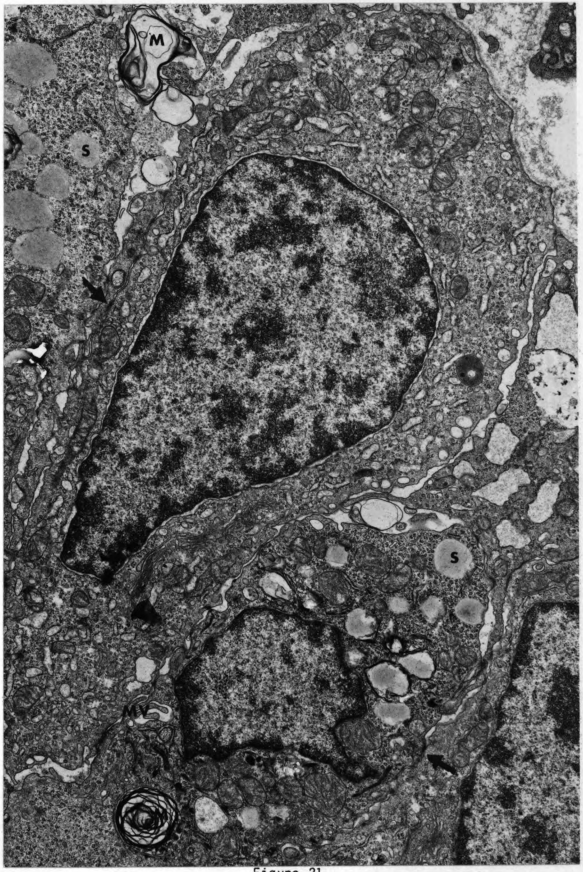


Figure 21

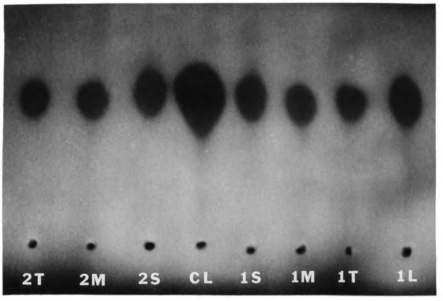


Figure 22. Starch gel electrophoresis of serum and tissue homogenates. Note the uniformly consistant distance of migration on the gel strip indicating a similar if not identical enzyme independent of the source. Tumor (2T), metastasis (2M), serum (2S) from tumor-bearing rat #2. Liver (CL) from control rat. Serum (1S), metastasis (1M), tumor (1T), liver (1L) from tumor-bearing rat #1. The lower dots are the point of origin.

DISCUSSION

Study I: Life Expectancy

The results of the life expectancy study indicated that this tumor progresses very rapidly and is highly predictable in behavior. This suggests that the R3230AC-L tumor could be a very useful animal model for studying mechanisms involved in neoplasia such as mechanisms of metastasis and involvement of the immune system with neoplasia. It could also be a useful animal model for testing the effects of potential antitumor agents as well as suspect tumor-promoting agents.

The loss of most rats near 6 weeks post-transplantation indicated that implantation into new recipients should probably be performed shortly before 6 weeks. Waiting beyond 6 weeks to transplant the tumor for continuing the line would increase the risk of losing the tumor bearing rats and, therefore, the tumor. At 4 weeks post-transplantation there often were not sufficient numbers of metastases, or they were not large enough for transplantation. Frequently at 6 weeks post-transplantation, the metastatic nodules were quite large with extensive areas of necrosis. Using these large nodules for transplantation could result in transplant failure because the tissue was too necrotic and non-viable. It would appear then, that 5 weeks might be the ideal transplantation time (Figure 23).

Clinical signs in recipient rats developed within 5 weeks of tumor implantation and progressed rapidly to severe cachexia, a moribund

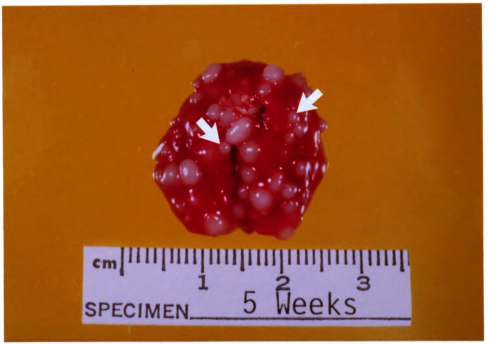


Figure 23. Lung with metastatic nodules of the R3230AC-L tumor 5 weeks after intrarenal implantation. Ideal size nodules for transplantation are indicated by the arrows.

condition and finally death at 6 weeks post-transplantation. The clinical signs included dyspnea, anorexia, abdominal pain and reluctance to move. The absence of internal body fat and lack of ingesta in the gastrointestinal tract suggested that starvation was contributory to the death of these animals. There was little functional lung tissue, the metastatic nodules having almost completely replaced the lung parenchyma. This observation suggested that asphyxiation was another major factor leading to the death of the rats.

Despite the aggressive growth of the primary tumor in the right kidney and rapid development of metastases in the lung, there was no evidence of hematogenous metastases to other tissues. This was a consistant feature in all studies reported here.

Study I: Time-Course Changes

Gross Pathologic Observations

The obvious agressive growth of the R3230AC-L tumor was further verified by the weight data. These data demonstrated an exponential increase in tumor weight which was coupled with a precipitous decrease in weight of the host kidney. By 6 weeks post-transplantation the right kidney, which was the site of tumor implantation, was no longer separable from the tumor and was generally unrecognizable except as a thin layer of compressed tubules only detectable through histologic examination. The loss of renal parenchyma was not caused by compression from the expanding tumor alone. This appeared grossly to be the operant mechanism at 2 weeks post-transplantation. However, renal tissue was observed surrounded by tumor cells and by 4 and 6 weeks post-trans-

plantation, it was increasingly difficult to separate tumor from the kidney. This indicated that the tumor had infiltrated into the kidney.

In addition to rapid growth within the kidney, the tumor demonstrated early tropism for the lung. Extrapolating from the observations that metastases were neither grossly nor microscopically apparent at 2 weeks post-transplantation, but were fairly numerous at 4 weeks post-transplantation lead to the conclusion that the metastases were probably established in the lung closer to the 2 week time frame than the 4 week time frame.

Adhesions that occurred between the right kidney and the liver in both tumor recipients and control rats could have been due to the mild hemorrhage which frequently occurred when the trochar and cannula were inserted beneath the right kidney capsule at the time of tumor implantation. A mild inflammatory reaction was also elicited by disruption of the renal capsule. The ensuing resorption of the clotted blood and resolution of the inflammatory reaction could have easily resulted in adhesion formation. Adhesions observed between intestines and tumor occurred only when the tumor was very large. These adhesions were not accompanied by constrictions of the intestinal lumen. Some control rats had a mild inflammatory reaction in the cortical region of the right kidney where the trochar and cannula might have been inserted.

Histopathologic Observations

Histologic progression of the tumor consisted of an increase in tumor size accompanied by larger more numerous areas of caseous necrosis. Also, accompanying increased tumor size was pressure necrosis of the bordering renal parenchyma and eventual invasion into the renal

tissue which ultimately resulted in total loss of the kidney. Initially the necrotic areas were confined to the central regions. Eventually necrotic changes were occurring at many levels within the tumor but necroses probably were all related to the tumor outgrowing its blood supply. It was surprising, however, that this aggressive, rapid growing tumor, which had so completely taken over the kidney, did not invade other adjacent tissues during its expansion within the abdominal cavity.

Histologic changes of metastases paralleled those seen in the primary tumor. Necrosis did occur in larger nodules, but not to the degree that was seen in the primary tumor. The largest of the metastatic nodules rarely exceeded 6mm in diameter. The lung provided excellent access to the circulation. Despite the fact that the nodules also appeared quite avascular, they were situated in an environment which provided them with easy access to the nutrients of the circulation. Being in a more propitious environment would also explain why cells of metastatic nodules appeared more metabolically active as evidenced by foamy expanded cytoplasm containing secretory products.

Liver was the only tissue in the proximity of the tumor in which neoplastic cells were observed. This appeared to be a bridging of the adhesion formed between the liver and tumor and the neoplastic cells extended into the hepatic parenchyma to a depth of only a few cells. Adhesions between intestines and tumor were not accompanied by histologic evidence of infiltration of malignant cells into intestinal tissues. When these adhesions did occur there had been extensive necrosis at the periphery of the tumor as well as deep within the neoplasm. (The necrosis was hypothesized to be related to the tumor outgrowing its blood supply.) The fibroblastic inflammatory response

which accompanied the necrotic change at the tumor surface might have inadvertently involved the intestine in adhesion formation.

Body and Organ Weights

The observation of a statistically significant elevation in terminal body weight of tumor bearing rats at 2 weeks post-transplantation compared to 2 week controls cannot be explained by an increase in individual organ weights. Possibly early in its development the tumor stimulates body growth. By 4 weeks post-transplantation there was no apparent difference in growth of the rats. However, at 6 weeks there was a notable decrease in the terminal weights of tumor-bearing rats. This is easily understood in light of clinical observations of inanition and cachexia. As a consequence of anorexia, there was catabolism of stored body fat and eventually body protein catabolism in an effort to supply nutritional needs of the animal.

The presence of metastatic tumors did not statistically affect lung weights until 6 weeks post-transplantation. At this stage normal lung parenchyma was nearly obliterated by tumors, and weights of the neoplastic lungs of the tumor-bearing rats were nearly 4 times those of control rats. At six weeks post-transplantation the left kidney was heavier in the tumor bearing rats when compared with control rats. This was apparently associated with compensatary hypertrophy for the failing right kidney. Histologic evidence of hypertrophy was not detected, however.

Hematology

While a slight decrease in total red blood cell count and increase in mean corpuscular hemoglobin were statistically significant in tumor recipients of the 2 week post-transplantation group, their biological significance was doubtful. Mean red blood cell count values of 7.33 x $10^6/\mu l$ and 7.85 x $10^6/\mu l$ for tumored and control rats respectively did not differ greatly from each other nor did they differ much from values in other groups. All of the mean red blood cell count values fell well within the normal range of 6.68 - 9.15 x $10^6/\mu l$ reported for the Fischer 344 rat (Mitruka and Rawnsley 1977).

A similar situation occurred with mean corpuscular hemoglobin (MCH) values. A biological interpretation would be difficult with the slight numerical difference between between 20.3 pg/red cell for tumor recipients and 19.57 pg/red cell for control animals. These values were also within the normal range reported as 17.0 to 21.8 pg/red cell in the Fischer rat (Mitruka and Rawnsley 1977). Since the decreased red blood cell count was used in the denominator for calculation of MCH it is not suprising that the MCH would show a significant difference also.

The statistically significant increased total white blood cell count in tumor recipients at 6 weeks post-transplantation was reflected primarily by increased neutrophils and slightly decreased lymphocyte numbers. While none of these values differed from reported normal ranges for Fischer 344 rats (Mitruka and Rawnsley 1977), there was a definite trend in tumor-bearing rats towards leukocytosis with neutrophilia. This trend was most likely associated with the presence of tissue necrosis in primary tumors and metastatic nodules.

Serum Clinical Chemistries

It is interesting to note that the first significant rise in alanine aminotransferase was observed at 4 weeks post-transplantation when metastasis was also first seen. Between the group means and among individual animals, a fairly good correlation was seen between total tumor tissue weights (primary tumor plus metastases) and elevation of enzyme activity (Figures 24 and 25). The rats with greater tumor tissue weights had higher serum levels of alanine aminotransferase. This would suggest some relationship between tumor size and enzyme elevation. Despite transplantation of tumor cells to adjacent liver in some rats, there was no good correlation with superficial hepatic implantation and activity of alanine aminotransferase in these animals. Further histologic and ultrastructural evidence of hepatic involvement was lacking. These observations would additionally support the conjecture that tumor per se was causing the elevation of the enzyme.

Significant elevation of serum aspartate aminotransferase in the 4 week and 6 week groups might be related to a number of organ systems or the tumor as it is not considered organ specific (Boyd 1962, Cornelius 1970 and Benjamin 1978). In the rat the greatest concentrations of this enzyme are in cardiac muscle and skeletal muscle (Boyd 1962). At 6 weeks post-transplantation rats were clinically in cardiac failure due to anoxia and increased resistance to circulation both caused by metastatic nodules in the lung. In addition, because of anorexia at this stage they were probably undergoing muscle catabolism to supply their metabolic needs. Cardiac failure and muscle catabolism might have caused the elevation of the serum aspartate aminotransferase.

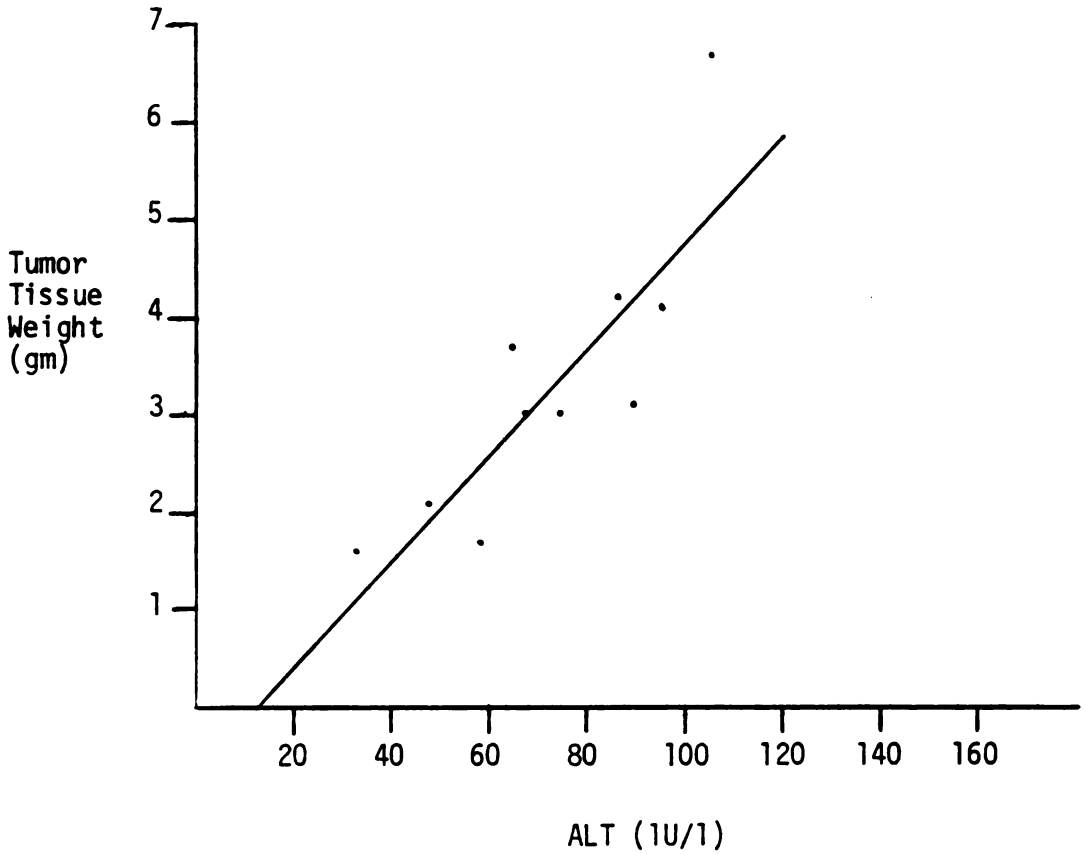


Figure 24. Correlation between total tumor tissue weight and serum alanine aminotransferase (ALT) at 4 weeks post-transplantation.

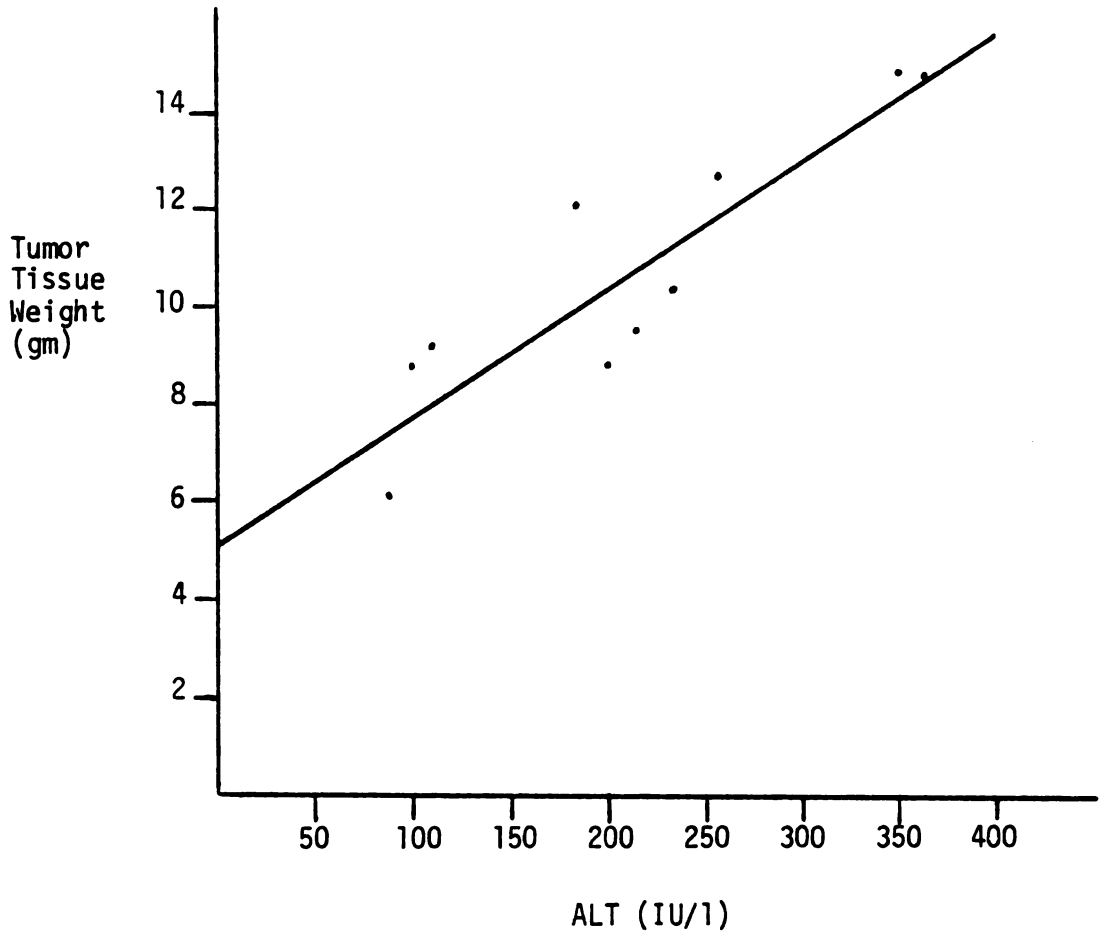


Figure 25. Correlation between total tumor tissue weight and serum alanine aminotransferase (ALT) at 6 weeks post-transplantation.

Decreased alkaline phosphatase values, observed in 4 and 6 week post-transplantation rats, were minimal and clinically not significant. According to Fishman et al., (1962) the major source of serum alkaline phosphatase in rats is the intestine. The mechanism whereby this enzyme reached the serum was linked to the ingestion of lipids (Lam and Mistilis 1973). Increased serum alkaline phosphatase occurred due to the normal fat content of the diet. A possible explanation for the decreased enzyme in tumor-bearing rats could be associated with the rats' decreased consumption of food causing a decrease in the lipid dependent intestinal form of serum alkaline phosphatase. According to Wolf (1979) decreased serum alkaline phosphatase is seen in hypophosphatasia, hypothyroidism, pernicious anemia and anticoagulated oxalated specimens. The mechanisms in hypothyroidism and pernicious anemia are related to the lack of thyroid hormone and vitamin B_{12} respectively which are required for osteoblastic function. It is the osteoblast which is considered one of the sources of serum alkaline phophatase. Rats used in these experiments were young, rapidly growing animals at the time of tumor implantation. Possibly there was a cessation of bone and body growth as the tumor reached a certain size resulting in a depletion of nutrients, hence a decrease in osteoblastic activity and thereby a decrease in serum alkaline phosphatase. Perhaps a combination decrease of intestinal and bone alkaline phosphatase was responsible for the decreased serum alkaline phosphatase.

Gamma glutamyl transpeptidase is not restricted to the liver. It has its highest activity in the kidney (Hardison 1979). In the rat it is not considered as good an indicator of hepatic disease as in the human because the rat liver contains only about one tenth the enzyme

activity that human liver contains (Shaw 1978). The elevations observed in the 4 and 6 week post-transplantation groups were small compared to the control groups and may not have biological significance. Gamma glutamyl transpeptidase was reported to increase in serum in association with various renal problems including infarction, neoplasia, transplant rejection and the nephrotic syndrome. It was also been reported to increase with chronic obstructive pulmonary disease. The mechanisms, however, were unexplained. Based upon those reports it was conjectured that the increased enzyme activity in serum from the 4 week post-transplantation group could have been related to the invasion of the tumor into the right kidney. In the 6 week group enzyme elevation could have been due to a combination of renal involvement and chronic obstructive pulmonary disease caused by numerous metastatic nodules.

Lactic dehydrogenase is another widely distributed enzyme in the body. Its elevation in serum of all groups of tumored rats could have been coming from a number of tissues. Elevations of total serum lactate dehydrogenase have been reported in a number of conditions which could be applicable to these rats including renal necrosis, pulmonary necrosis, hepatic congestion, cardiac failure, anoxic tissue damage and neoplasia (Benjamin 1978, Dito 1979).

Although statistically, there was a significant difference in leucine aminopeptidase in the 6 week group of tumored rats, the clinical significance was dubious. There was a difference of only 2 international units per liter between tumor recipients and the controls. In clinical medicine, enzyme levels are interpreted as normal within a range of values and elevations are not considered meaningful unless there is a several fold increase above normal control values. This

enzyme is present in several tissues besides the liver including duodenum, small intestine, renal tubules, pancreas, testes and uterus (Wolf 1979). In this instance the observed elevation could have been due to involvement of the kidney.

Statistically significant elevations of aspartate aminotransferase, gamma glutamyl transpeptidase, lactic dehydrogenase and leucine aminopeptidase could be explained in terms of mechanisms other than hepatic involvement. This was supported by the absence of any visible deviations from control livers at the gross and microscopic levels. Serum elevation of alanine aminotransferase could not be explained in terms of other known mechanisms. The hypothesis that the source was the R3230AC-L tumor was tested further by performing enzyme analyses on tissue homogenates.

Study II: Enzyme Activity in Liver and Tumor Homogenates

This study assumed that if the tumor does contain alanine aminotransferase, it was almost entirely in the soluble cytosolic form as was true for the enzyme in rat liver. Analyses of tumor tissue homogenates confirmed the presence of soluble enzyme in the primary tumor and metastases. Those activities observed for the soluble enzyme in the tumor and metastases should be considered in relation to the tissues contribution to the total body weight. Even though neoplastic tissues contained one fifth to one third the activity of enzyme in the liver, their combined weight was 3 times that of the liver. It is feasible that this amount of tissue containing that much enzyme activity could have made a significant contribution to the serum values. It should be kept in mind that the actual activity in the

tumor and metastases might have been even higher than measured if they also contained an appreciable amount of membrane bound enzyme since only cytosolic alanine aminotransferase was measured.

The mechanism of release of enzyme from the tumor is not known. One of the mechanisms for release of alanine aminotransferase from the liver into serum is increased cell membrane permeability. This is an early degenerative change, which allows the cytosolic enzyme to escape into extracellular fluids, including serum. Because the tumor tissues were undergoing degenerative changes, it was speculated that the alanine aminotransferase which they contained was escaping into extracellular fluids by a similar mechanism of increased cell membrane permeability.

The histologic appearance of tumor and metastases reconfirmed the consistancy noted in the parent tumor line. This tumor had maintained its cellular characteristics over several transplant generations. The scope of this entire study encompassed eleven transplant generations and morphologically the tumor appeared similar from one generation to the next.

At the gross, microscopic and ultrastructural levels no lesions or differences from controls were observed in livers of tumor bearing rats. The lack of changes at the ultrastructural level further supported the hypotheses of previous studies, that elevated alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transpeptidase, lactate dehydrogenase and leucine aminopeptidase were probably coming from sources other than the liver.

Electron Microscopy

Ultrastructural features of metastatic tumor cells suggested an increased level of metabolic activity compared to primary tumor cells. There were more Golgi appartus, endoplasmic reticulum and secretory products in metastatic tumor cells compared to primary tumor cells. Because the metastatic cells were in a better position for obtaining oxygen and nutrients from the blood, they would have been more capable of carrying on secretory functions which might account for the higher levels of alanine aminotransferase. In constrast, the primary implant was poorly vascularized, probably in a constant state of hypoxia, and, therefore, less able to carry on secretory activities.

Study III: Electrophoresis of Serum and Liver and Tumor Homogenates

Because electrophoresis is considered one of the best methods for the detection of the purity of an enzyme and for the detection of the presence of isoenzymes (Anderson and Green 1967 and Wiseman and Gould 1971), it was chosen as the method for comparing alanine aminotransferase from different tissue sources. The uniformity of migration of the alanine aminotransferase, from tumor tissues compared to livers of control rats and tumor-bearing rats indicated that the enzymes from the different sources were very similar if not identical. Isoenzymes were not detected in any of the tissues tested. This was expected for the hepatic tissue. It was surprising, however, to have discovered such similarity between enzymes from the liver and mammary neoplasm, two such different tissue types. A teleological explanation for the presence of alanine aminotransferase in the R3230AC-L mammary adenocarcinoma was not discovered.

SUMMARY

The R3230AC-L tumor (Research number 3230 adenocarcinoma-lung) was responsible for elevated serum levels of the liver specific enzyme alanine aminotransferase in tumor-bearing rats. The enzyme from the mammary tumor was electrophorecticly identical to the enzyme produced by the liver. Other liver-specific clinical pathologic tests did not indicate any hepatic involvement in tumor bearing rats. The absence of hepatic involvement was further supported by gross, microscopic and ultrastructural examinations of liver tissues from the tumor-bearing rats.

Study I: Life Expectancy and Time-Course Study

There was a sequential rise in serum alanine aminotransferase which paralleled the growth and metastasis of the tumor. The rising values terminated with death of the animals at approximately 6 weeks post-transplantation. Serum clinical chemistries and hematologic values supported the gross and microscopic findings except for the elevated alanine aminotransferase. There was no indication of any hepatic involvement which would ordinarily be considered the source of alanine aminotransferase in serum.

Study II: Enzyme Activity in Liver and Tumor Homogenates

Alanine aminotransferase was identified in homogenates of tumor tissues. This supported the premise that the tumor was the source of serum elevations of the enzyme. Although unit values per gram of tissue were much lower in the tumor, (approximately one-fourth of the

liver) the percentage of total body weight attributed to tumor tissues was much greater than the liver (approximately 3 times the liver percentage). There was no evidence of hepatic damage. Tumor tissues, on the other hand, were undergoing necrosis, and the degenerative changes could have allowed the escape of alanine aminotransferase from those tissues into the circulation.

Study III: Electrophoresis of Serum and Liver and Tumor Homogenates

The similarity in electrophoretic patterns indicated that tumor alanine aminotransferase was similar (if not identical) to cytosolic alanine aminotransferase from rat liver. This raised a number of unanswered questions including the explanations for a non-hepatic tissue needing or using this particular enzyme when the enzyme is not known to exist in significant amounts in any rat tissue other than liver.

Applications of the R3230AC-L Data

These studies have identified and characterized a new research tool for the study of neoplastic disease. The R3230AC tumor has already been recognized as a valid and valuable animal model of certain breast cancers in humans, and it is very likely that its subline, the R3230AC-L tumor, would have similar value. Furthermore, association of serum alanine aminotransferase levels with R3230AC-L tumor growth might have implications of diagnostic or prognostic importance with respect to similar types of neoplasia in man.

The R3230AC-L tumor could be an excellent animal test system for screening anti-tumor agents. Serum alanine aminotransferase levels in tumor bearing rats could be utilized as a clinical measure for the estimation of tumor developoment in those studies of anti-tumor agents.

More work is needed for delineating the mechanisms and metabolism involved in this unique enzyme association with the mammary tumor. When attained, this knowledge will contribute to further understanding of malignant transformations.

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APPENDICES

Appendix 1. Media, Dialysis and Storage Effects upon Alanine Aminotransferase and Hemoglobin in Liver and Kidney Homogenates

Purpose

This study was designed to test the effects of medium, dialysis and storage upon the levels of alanine aminotransferase and hemoglobin in rat tissue homogenates. Hemoglobin levels were determined in homogenates as an estimate of the degree of blood contamination.

The choice of medium for homogenization of tissues and the subsequent analysis of the homogenate's constituents is a highly empirical matter (Morton 1955, Potter 1955, Moulé 1963, deDuve, C. 1967 and Hess and Brand 1974). Much depends upon the stabilily of the substance being analyzed in the homogenate. In general, high molecule weight compounds such as enzymes are not as labile as low molecular weight metabolites (Hess and Brand 1974). The media tested in this study were: sucrose 0.25 molar, saline 0.15 molar and Triton X-100 0.5% (volume/volume). Several investigators had advocated these media for homogenization (Morton 1955, Potter 1955, Schneider 1961, Moulé and Chauveau 1963, Potter 1964, deDuve 1967 and Hess and Brand 1974). While saline was generally used for the initial rapid chilling of tissue samples, sucrose was preferred because, unlike ionic media, it did not promote aggregation of the subcellular components. Where disruption of cellular membrane systems was desirable, Triton X-100 was used. The lack of buffering capacity in these media caused no problems as long as the homogenates were kept cold (Schneider 1961, Moulé and Chauveau 1963).

Because dialysis will remove low molecular weight substances which may interfere with enzyme activities and because the homogenates consisted of a conglomerate of disrupted cellular components, it was thought that dialyzing the homogenates might increase the measured activity of alanine aminotransferase and the measured amount of hemoglobin.

It is frequently necessary to hold samples for varying periods of time before analysis. Reports differ as to the stability of alanine aminotransferase held refrigerated or frozen (Swick et al., 1965, Dade Diagnostics 1977 and Benjamin 1978). This study tested the effects of storage of homogenates under refrigeration at 5 - 10°C for up to 8 days and the effects of freezing at -60°C for up to 1 month.

Materials and Methods

Animals

A total of 15 female Fischer[a] rats weighing between 90 and 135 grams were used. They were randomly assigned 3 each to a group. Within the group each rat contributed 3 pieces of liver and 3 pieces of kidney to be placed one piece to each of 3 medias, 0.15 molar saline, 0.25 molar sucrose and 0.5% (v/v) Triton X-100.[b] The three pieces in each media, representing one sample from each rat within the group, were combined as one homogenate. So that each group had a total of 6 homogenates: 3 different medias for the liver and 3 different medias for the kidney. Each homogenate for a given media was divided into 14

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[a]Fischer 344/NUpj, The Upjohn Company, Kalamazoo, Michigan.

[b]Triton X-100, Eastman Kodak Company, Rochester, New York.

samples each of which were placed in a factorial arrangement of dialysis or no dialysis and 7 storage time periods: 1, 3 or 8 days unfrozen or 1, 2, 3 or 4 weeks frozen. At the end of the assigned time period, the samples were analyzed twice for alanine aminotransferase and hemoglobin.

Homogenization Procedure

Each rat was weighed. To facilitate blood collection the rat was sedated with carbon dioxide vapor from dry ice[c] and blood was collected via cardiac puncture. The rat was killed by exsanguination. Part of the blood sample was placed in a vial containing ethylenediamine tetra-acetic acid[d] for hemoglobin determination with an automated electronic analyzer.[e] The other part of the blood sample was placed in a silicone coated glass vial and was allowed to clot. After centrifugation at 2000 revolutions per minute for 15 minutes, the serum was removed from the clotted sample. The serum samples for each group were pooled for analysis for alanine aminotransferase. The analysis was according to the method of Wroblewski and LaDue (1956) utilizing an automated centrifical analyzer.[f]

The abdominal cavity was opened with a ventral midline incision. The liver and kidneys were quickly removed, weighed and then placed in

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[c]Cardox®, Division of Chemetron Corporation, Countryside, Illinois.

[d]Vacutainer, Becton, Dickinson and Company, Rutherford, New Jersey.

[e]Coulter S., Coulter Electronics, Inc., Hialeah, Florida.

[f]Centrifichem, Union Carbide Corp., Rye, New York.

cold 0.15 molar saline. All tissue samples were maintained in an ice bath at all times to minimize enzymatic degredation. Several thin slices were removed from the liver and from the cortex of the kidneys and were placed in fresh containers of saline in order to further facilitate the removal of blood from the tissues. Three slices of liver tissue (approximating 100 mg each) were blotted on a piece of gauze, and weighed individually. One piece was placed in each of the three medias in a 10 ml volumetric flask, 0.15 molar saline, 0.25 molar sucrose or 0.05% Triton X-100. Three slices from the kidney were handled similarly. These procedures were repeated with each rat. The volumetric flasks were brought up to volume with the appropriate solution. The liver tissues from the three rats in a given media were homogenized together in a Potter-Elvehjen glass homogenizer[g] fitted with a motor driven[h] teflon pestle at 1000 revolutions per minute for 2 one-minute periods according to the method of Schneider (1961). The samples were transferred to centrifuge tubes and were spun in a pre-cooled refrigerated centrifuge (2-4°C)[i] for 15 minutes at 1500 x g. The supernatant was decanted and re-centrifuged in a high speed refrigerated centrifuge (2-4°C)[j] at 39,300 x g for one hour. The final supernatant

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[g]A. H. Thomas Company, Philadelphia, Pennsylvania.

[h]Tri-R Stir-R Model K41, Tri-R Laboratory Instruments, Inc., New York, New York.

[i]Beckman Model TJ-6, Beckman Instruments, Inc., Palo Alto, California.

[j]Sorvall Superspeed, Model RC2-B, Dupont Instruments, Biomedical Division, Newtown, Connecticut.

was divided into 14 vials, half of which were to be dialyzed at the time periods mentioned in the previous section and the remaining 7 which were not to be dialyzed. The samples which were stored unfrozen were maintained in a refrigerator at 5-10°C. The samples which were stored frozen were maintained at -60°C.

Dialysis Procedure

Dialysis was performed according to the method of McPhie (1971). Two milliliters of the sample to be dialyzed were placed in size 8 dialysis tubing[k] which was then suspended in a one liter contianer of distilled water. The sample was dialyzed for 18 hours. At the end of that time the final volume of sample was recorded and then it was analyzed for alanine aminotransferase and hemoglobin using an automated centrifical analyzer. A control serum sample[l] was also analyzed as an external control of consistancy in the results. A method for alanine aminotransferase was according to Wroblewski and LaDue (1956). The method for hemoglobin was according to Standefer and Vanderjagt (1977). The purpose of the hemoglobin analysis was for factoring out that part of the tissue alanine aminotransferase which was due to blood contamination (Hess and Brand 1974).

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[k]Union Carbide Corporation, Films, Packaging Division, Chicago, Illinois.

[l]Moni-Trol I, Dade Division, American Hospital Supply Corporation, Miami, Florida.

Statistical Analysis

The data were analyzed using least squares analysis of variance (Steel and Torrie 1960). The comparisons consisted of media (the saline, sucrose or Triton X-100), dialysis versus no dialysis, storage time (1, 3 or 8 days unfrozen or 1, 2, 3 or 4 weeks frozen), group (the 5 three-rat groups) and several of the two and three-way interactions. Pairwaise comparisons of the means were made using the Least Significant Difference Test. Significance was determined at the .05 level.

Results and Discussion

The statistical data for main effects are summarized in Tables 6, 7, and 8. Statistical differences were detected. Their biological significance, however, is questionable particularly with respect to the alanine aminotransferase levels where, for example, the difference between the mean liver ALT in Triton X-100 and that in sucrose was only a matter of 1.7 IU/gm of tissue. The differences were even smaller when comparing the mean alanine aminotransferase levels in the kidney. When dealing with enzymes in clinical pathology one generally looks at ranges of activity. The statistically significant differences with respect to hemoglobin levels in the various media would appear to be biologically meaningful. The sucrose media gave consistantly lower results for the hemoglobin determinations. The Triton X-10 gave much higher results for the liver hemoglobin determination but not for the kidney. Because the liver is one of the sites of hemoglobin breakdown, there may normally be more hemoglobin present and the action of Triton X-100 upon cell membranes may be releasing more of the hemoglobin than the mechanical effect of homogenization alone. The measured amount of hemoglobin would

Table 6. The mean values of liver and kidney alanine aminotransferase and hemoglobin with different media

	Media		
	Saline	Sucrose	Triton X-100
Liver ALT(IU/gm)	24.4 ab	24.5 b	22.8 a
Liver Hgb(mg/L)	33.0 b	7.6 a	50.5 c
Kidney ALT(IU/gm)	2.4 b	1.2 a	1.6 c
Kidney Hgb (mg/L)	39.0 b	16.5 a	37.5 b

Table 7. The mean values of liver and kidney alanine aminotransferase and hemoglobin with and without dialysis

	Dialysis	
	Yes	No
Liver ALT(IU/gm)	24.1	23.7
Liver Hgb(mg/L)	30.7	29.9
Kidney ALT(IU/gm)	1.3	1.5
Kidney Hgb(mg/L)	29.6 a	32.3 b

Means in a horizontal row with no common letters (abc) are significantly different at or near the .05 level.

Table 8. The mean values of liver and kidney alanine aminotransferase and hemoglobin with different storage times

	Time						
	1 day	3 days	8 days	1 week	2 weeks	3 weeks	4 weeks
Liver ALT (IU/gm)	24.4	23.5	23.8	23.8	24.3	23.7	24.0
Liver Hgb (mg/R)	30	29	32	31	30	30	31
Kidney ALT (IU/gm)	1.4 abc	1.5 bc	1.4 abc	1.4 a	1.4 ab	1.5 c	1.4 a
Kidney Hgb (mg/L)	30	30	32	30	31	32	31

Means in a horizontal row with no common letters (abc) are significantly different at or near the .05 level.

then include stored liver hemoglobin in addition to that present from blood remaining in the tissue.

Although a statistical difference was detected in the results of dialysis on kidney hemoglobin levels, it is doubtful that this difference is biologically significant as the numerical difference between the two means is so small (2.7 mg/L).

Where statistically significant interaction effects were detected, there was no consistant pattern. The differences were so small that none of the interaction effects could be considered biologically meaningful (Tables 9, 10, 11, 12, 13, and 14).

In summary this study detected no major effects of media, dialysis or storage upon the levels of alanine aminotransferase in the supernatant fraction from the homogenization of liver or kidney tissues. The only major effect noted was that of media choice upon hemoglobin levels with 0.25 molar sucrose giving consistantly lower values for both liver and kidney homogenates and Triton X-100 giving higher values for liver homogenates.

The sodium chloride media at 0.15 molar concentration seemed to be the media of choice for the determination of alanine aminotransferase activity in liver and kidney homogenates. Besides producing the poorest results for hemoglobin levels, the sucrose media was a problem to store and would easily become contaminated. The Triton X-100 was more difficult to work with in that a persistant froth quickly developed during any agitation of the solution.

Table 9. Kidney ALT(IU/gm): media and dialysis interaction (p=.000)

Dialysis	Saline	Sucrose	Triton X-100
Yes	1.5	1.2	1.3
No	1.4	1.2	2.0

Table 10. Kidney ALT(IU/gm): dialysis and storage interaction (p=.000)

Time	Dialysis - yes	Dialysis - no
1 day	1.2	1.6
3 days	1.3	1.7
8 days	1.3	1.6
1 week-frozen	1.3	1.4
2 weeks-frozen	1.4	1.4
3 weeks-frozen	1.5	1.5
4 weeks-frozen	1.3	1.4

Table 11. Liver ALT(IU/gm): media and dialysis interaction (p=.045)

Dialysis	Saline	Sucrose	Triton X-100
Yes	23.3	25.2	23.8
No	25.5	23.8	21.8

Table 12. Liver hemoglobin (mg/l): dialysis and storage interaction (p=.008)

Time	Dialysis - yes	Dialysis - no
1 day	23.6	25.1
3 days	23.1	24.0
8 days	24.0	23.5
1 week-frozen	24.6	22.9
2 weeks-frozen	23.4	15.1
3 weeks-frozen	25.2	22.2
4 weeks-frozen	24.6	23.3

Table 13. Kidney hemoglobin (mg/l): media and dialysis interaction (p=.002)

Dialysis	Saline	Sucrose	Triton X-100
Yes	36.2	16.1	36.6
No	41.8	16.9	38.4

Table 14. Kidney hemoglobin (mg/l): dialysis and storage interaction (p=.032)

Time	Dialysis - yes	Dialysis - no
1 day	27.2	33.2
3 days	27.9	31.8
8 days	30.9	33.4
1 week	28.8	31.9
2 weeks	30.8	31.6
3 weeks	32.2	32.6
4 weeks	29.5	31.9

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Appendix 2. Reagents for Staining ALT Electrophoresis

Contents of GPT 10 vial[a]	Amount	Concentration
Sodium phosphates	3.0 mmoles	300 mM
D, L-alanine	2.42 mmoles	242 mM
β-NADH	7.0 μmoles	0.7mM
LDH	>40 U(25°C)	>4.0 U/ml
(25°C)		
α-Ketoglutarate	246 μmoles	24.6mM
Non-reactive stabilizers		

[a]Bio-Dynamics/bmc, Indianapolis, Indiana

Appendix 3. Study 1, Group 1 - Body and Organ Weights (grams) 2 Weeks Post-transplantation

Animal Number	Initial Body Weight	Final Body Weight	Lung	Liver	Left Kidney	Right Kidney	Tumor
Tumored							
3-1	78	110	0.588	5.013	0.576	0.495	0.131
11-2	85	122	0.628	5.113	0.570	0.458	0.214
12-6	66	109	0.640	4.658	0.565	0.532	0.455
10-7	65	109	0.553	4.739	0.576	0.523	0.330
9-4	83	111	0.523	4.101	0.496	0.449	0.225
8-10	80	107	0.535	3.840	0.526	0.481	0.300
4-7	68	107	0.604	4.500	0.553	0.488	0.157
6-9	87	113	0.672	4.260	0.546	0.547	0.396
5-5	69	107	0.628	4.629	0.571	0.496	0.216
2-10	103	130	0.662	5.621	0.650	0.607	0.284
Mean[a]	78.4±4.2	112.5±2.7	0.60±0.00	4.65±0.16	0.56±0.02	51±0.00	0.27±0.03
Controls							
15-2	59	95	0.558	3.667	0.465	0.448	
16-8	76	115	0.701	4.721	0.670	0.585	
13-5	77	97	0.524	3.856	0.505	0.491	
18-9	96	111	0.698	4.715	0.574	0.572	
17-5	57	93	0.562	4.517	0.507	0.512	
Mean[a]	73.0±5.9	102.2±3.8	0.61±0.03	4.30±0.23	0.54±0.02	0.52±0.02	

[a] Expressed as mean ± SEM

Appendix 4. Study 1, Group 2 - Body and Organ Weights (grams) 4 Weeks Post-transplantation

Animal Number	Initial Body Weight	Final Body Weight	Lung	Liver	Left Kidney	Right Kidney	Tumor
Tumored							
5-1	84	128	0.631	5.323	0.632	0.515	0.991
1-4	93	129	0.751	6.223	0.662	0.574	2.294
2-6	67	141	1.080	7.421	0.938	0.495	5.632
9-2	81	143	0.895	6.312	0.720	0.555	2.136
12-10	63	124	0.930	6.311	0.751	0.479	2.777
7-2	67	110	0.652	4.632	0.631	0.463	1.430
3-2	75	144	0.817	6.422	0.678	0.625	0.884
10-6	51	138	1.024	6.490	0.881	0.615	3.131
11-3	72	128	0.808	6.032	0.787	0.432	3.260
6-8	82	135	0.990	5.327	0.715	0.670	2.145
Mean[a]	73.5±4.0	132.0±3.1	0.86±0.03	6.04±0.23	0.74±0.03	0.55±0.02	2.47±0.44
Controls							
16-10	64	127	0.803	5.624	0.682	0.646	
14-7	66	135	0.765	6.429	0.720	0.686	
13-1	89	122	0.703	5.333	0.650	0.621	
18-8	95	143	0.925	6.511	0.688	0.735	
17-4	84	130	0.758	5.198	0.673	0.622	
Mean[a]	79.6±5.7	131.4±4.4	0.79±0.06	5.82±0.33	0.68±0.04	0.66±0.03	

[a]Expressed as mean ± SEM

Appendix 5. Study I, Group 3 - Body and Organ Weights (grams) 6 Weeks Post-transplantation

Animal Number	Initial Body Weight	Final Body Weight	Lung	Liver	Left Kidney	Right Kidney	Tumor
Tumored							
6-7	89	126	4.785	5.698	0.702	-	4.832
2-9	94	134	2.444	5.001	0.702	-	3.674
8-8	84	96	7.212	3.727	0.569	-	4.855
12-7	87	134	8.051	5.971	0.728	-	6.727
8-6	92	115	8.438	4.898	0.688	-	6.492
1-1	95	120	8.542	5.424	0.690	0.352	4.941
11-1	66	129	4.460	5.172	0.686	0.426	3.615
7-3	65	105	7.641	4.362	0.624	0.363	4.443
4-8	69	142	3.882	5.334	0.700	-	3.866
12-8	86	136	4.777	6.512	0.854	-	7.931
Mean[a]	82.7±3.9	123.7±5.2	6.02±0.58	5.21±0.26	0.69±0.02	0.38±0.03	5.14±0.46
Controls							
16-7	74	152	0.873	6.412	0.694	0.681	
18-6	87	153	0.752	5.981	0.730	0.695	
18-7	72	161	0.840	6.685	c	0.672	
16-9	50	110	0.606	4.455	0.569	0.561	
17-3	65	150	0.764	5.872	0.693	0.669	
Mean[a]	69.6±5.5	145.2±7.4	0.77±0.82	5.88±0.36	0.67±0.03	0.66±0.02	

[a]Expressed as mean ± SEM

Appendix 6. Study I, Group 1 - Hematologic Values 2 Weeks Post-transplantation

Animal Number	WBC ($x10^3/\mu l$)	RBC ($x10^6/\mu l$)	Hgb (g/dl)	PCV (%)	MCV (fe)	MCH (pg)	MCHC (g/dl)
Tumored							
3-1	6.9	7.18	15.1	47.3	66	20.9	31.9
11-2	2.7	7.29	14.8	47.1	65	20.2	31.3
10-7	7.6	7.21	14.8	48.1	67	20.5	30.8
8-10	5.5	7.40	14.5	49.3	67	19.5	29.2
4-7	5.1	6.99	14.2	46.0	66	20.3	30.8
5-5	9.6	7.49	15.3	47.4	63	20.4	32.3
2-10	8.8	7.73	15.8	49.2	64	20.4	32.1
Mean[a]	6.6±2.4	7.33±0.24	14.9±0.5	47.8±1.2	65.4±1.5	20.3±0.4	31.2±1.1
Controls							
15-2	5.5	7.77	15.2	49.8	64.0	19.7	30.7
13-5	4.7	8.13	49.0	61.0	19.5	32.3	0.0
17-5	2.5	7.65	15.0	48.5	64.0	19.5	30.8
Mean[a]	4.2±1.6	7.85±0.25	15.4±0.5	49.1±0.7	62.0±1.7	19.6±0.1	31.3±0.9

[a]Expressed as mean ± SEM

Appendix 6. Continued

Animal Number	Non-Seg (%)	Seg (%)	Lymph (%)	Mono (%)	Eo (%)	Baso (%)	NRBC (/100WBC)	Atypical RBC(/100WBC)
Tumored								
3-1	0	8	90	1	1	0	1	0
11-2	0	17	83	0	0	0	0	0
10-7	0	16	84	0	0	0	5	0
8-10	0	8	89	0	3	0	0	0
4-7	0	9	81	0	0	0	1	0
5-5	0	9	91	0	0	0	0	0
2-10	0	24	75	0	1	0	1	0
Mean[a]	0	13.0±6.2	83.7±5.7	0.1±0.4	0.7±1.1	0	1.1±1.8	0
Controls								
15-2	0	13	87	0	0	0	0	0
13-5	7	93	0	0	0	0	0	0
17-5	0	18	81	0	1	0	0	0
Mean[a]	0	12.7±5.5	87.0±6.0	0	0.3±0.6			

[a]Expressed as mean ± SEM

Appendix 7. Study I, Group 2 - Hematologic Values 4 Weeks Post-transplantation

Animal Number	WBC ($x10^3/\mu l$)	RBC ($x10^6/\mu l$)	Hgb (g/dl)	PCV (%)	MCV (fe)	MCH (pg)	MCHC (g/dl)
Tumored							
5-1	8.4	8.21	16.0	46.4	57	19.4	34.5
1-4	9.3	7.71	14.6	43.1	56	18.8	33.7
2-6	6.0	7.18	13.9	42.7	60	19.2	32.3
9-2	8.6	7.55	14.6	44.0	59	19.3	33.0
12-10	13.2	4.61	9.0	29.4	64	19.5	30.5
7-2	8.8	7.82	15.2	46.1	60	19.3	32.8
3-2	9.4	7.52	14.7	45.1	61	19.4	32.4
10-6	9.3	7.29	14.7	44.5	62	20.1	33.0
11-3	10.2	7.52	14.4	45.1	61	19.0	31.7
6-8	7.2	7.85	14.9	45.7	59	18.9	32.5
Mean[a]	9.0±1.9	7.33±1.00	14.2±1.9	43.2±5.0	59.9±2.3	19.2±0.4	32.6±1.1
Controls							
16-10	7.4	8.06	15.7	48.6	61	19.4	32.2
14-7	5.9	8.02	15.8	48.5	61	19.6	32.4
13-1	6.5	8.91	16.5	50.5	57	18.4	32.6
18-8	7.0	8.25	15.8	47.7	59	19.1	33.1
17-4	7.0	8.18	15.6	47.5	59	19.0	32.8
Mean[a]	6.8±0.6	8.9±0.4	15.9±0.4	48.6±1.2	59.4±1.7	19.1±0.5	32.6±0.4

[a]Expressed as mean ± SEM

Appendix 7. Continued

Animal Number	Non-Seg (%)	Seg (%)	Lymph (%)	Mono (%)	Eo (%)	Baso (%)	NRBC (/100WBC)	Atypical RBC(/100WBC)
Tumored								
5-1	0	18	81	1	0	0	1	0
1-4	0	16	81	0	3	0	1	0
2-6	0	22	75	0	3	0	0	0
9-2	0	24	76	0	0	0	1	0
12-10	0	33	66	0	1	0	7	0
7-2	0	18	81	0	1	0	0	0
3-2	0	34	65	0	1	0	1	0
10-6	0	24	75	0	1	0	0	0
11-3	1	37	62	0	0	0	0	0
6-8	0	28	72	0	0	0	0	0
Mean[a]	0.1	25.4±7.4	73.4±7.0	0.1	1.0±1.6	0	1.1±2.1	0
Controls								
16-10	0	14	85	1	0	0	0	0
14-7	0	17	81	0	2	0	0	0
13-1	0	12	86	0	2	0	0	0
18-8	0	20	77	0	3	0	0	0
17-4	0	8	92	0	0	0	0	0
Mean[a]	0	14.2±4.6	84.2±5.6	0.2±0.4	1.4±1.3	0	0	0

[a]Expressed as mean ± SEM

Appendix 8. Study I, Group 3 - Hematologic Values 6 Weeks Post-transplantation

Animal Number	WBC ($x10^3/\mu l$)	RBC ($x10^6/\mu l$)	Hgb (g/dl)	PCV (%)	MCV (fe)	MCH (pg)	MCHC (g/dl)
Tumored							
6-7	10.6	7.95	15.7	44.1	56	19.7	35.5
2-9	10.9	7.46	15.0	42.2	57	19.9	35.3
8-8	8.2	8.48	17.0	47.9	57	19.8	35.3
12-7	9.8	7.86	15.1	45.0	58	19.1	33.4
8-6	10.9	8.10	15.6	46.1	58	19.1	33.7
1-1	9.0	7.84	14.6	43.5	56	18.5	33.5
11-1	8.8	7.92	15.2	46.3	59	19.1	32.7
7-3	8.4	7.61	15.4	46.4	62	20.1	33.0
4-8	10.0	8.18	15.7	47.8	59	19.1	32.8
12-8	11.7	7.66	14.2	43.1	57	18.4	32.7
Mean[a]	9.8±1.2	7.91±0.30	15.4±0.8	45.2±1.2	57.9±1.8	19.3±0.6	33.8±1.1
Controls							
16-7	8.5	8.07	15.7	46.1	58	19.3	33.8
18-6	6.3	8.31	15.8	48.1	59	18.9	32.7
18-7	6.4	8.32	16.3	51.2	62	19.5	31.8
16-9	6.5	8.29	16.4	49.1	60	19.6	33.2
17-3	4.6	7.55	14.5	42.9	57	19.1	33.6
Mean[a]	6.5±1.4	8.11±0.33	15.7±0.8	47.5±3.2	59.2±1.9	19.3±0.3	33.0±0.8

[a]Expressed as mean ± SEM

Appendix 8. Continued

Animal Number	Non-Seg (%)	Seg (%)	Lymph (%)	Mono (%)	Eo (%)	Baso (%)	NRBC (/100WBC)	Atypical RBC(/100WBC)
Tumored								
6-7	1	47	51	0	1	0	2	0
2-9	0	28	71	0	1	0	2	0
8-8	0	77	21	2	0	0	7	0
12-7	2	39	57	0	2	0	2	1
8-6	0	64	34	2	0	0	9	0
1-1	0	79	19	1	1	0	7	0
11-1	0	33	66	0	1	0	0	0
7-3	0	83	16	0	1	0	9	0
4-8	0	37	61	0	2	0	1	0
12-8	0	38	60	1	1	0	1	0
Mean[a]	0.3±0.7	52.5±21.1	45.6±21.0	0.6±0.84	1.0±0.7	0	4.0±3.6	0.1±0.3
Controls								
16-7	0	12	87	1	0	0	0	0
18-6	0	24	73	1	2	0	0	0
18-7	0	22	77	0	1	0	1	0
16-9	0	15	84	0	1	0	0	0
17-3	0	7	93	0	0	0	0	0
Mean[a]	0	16.0±7.0	82.8±8.0	0.4±0.6	0.8±0.8	0	0.2±0.4	0

[a]Expressed as mean ± SEM

Appendix 9. Study I, Group 1 - Clinical Chemistry Values 2 Weeks Post-transplantation

Animal Number	ALT IU/L	APT IU/L	SDH IU/L	Alk. Phos. IU/L	GGT IU/L	LDH IU/L
Tumored						
3-1	14	111	11	375	2	-
11-2	13	81	1	314	12	637
12-6	16	111	3	341	8	481
10-7	16	75	9	345	5	612
9-4	13	67	3	294	6	653
8-10	16	75	3	297	8	485
4-7	11	70	3	321	6	458
6-19	16	64	6	278	5	276
5-5	13	57	6	330	9	222
2-10	18	51	3	166	9	214
Mean[a]	14.6±.8	76.2±5.6	4.8±1.0	316.1±11.2	7.0±0.8	448.7±49.0
Controls						
15-2	13	65	7	399	8	174
16-8	13	67	7	344	3	198
13-5	20	81	12	318	6	276
18-9	13	66	4	308	9	228
17-5	11	58	3	383	8	102
Mean[a]	14.0±1.2	67.4±7.8	6.6±1.5	350.4±15.8	6.8±1.2	195.6±67.8

[a]Expressed as mean ± SEM

Appendix 9. Continued

Animal Number	D. Bili. mg%	T. Bili. mg%	T. Prot. gm%	Alb. gm%	BUN mg%
3-1	0.25	-	5.3	3.9	22
11-2	0.08	0.34	5.4	3.7	22
12-6	0.03	0.13	5.1	3.6	18
10-7	0.06	0.20	5.4	3.7	23
9-4	0.05	0.20	5.0	3.5	26
8-10	0.06	0.11	5.0	3.5	21
4-7	0.03	0.15	5.1	3.6	20
6-19	0.17	0.20	5.3	3.7	21
5-5	0.11	0.15	5.8	4.0	18
2-10	0.08	0.25	5.6	3.9	21
Mean[a]	0.09±0.02	0.19±0.02	5.3±0.1	3.7±0.0	21.1±0.7
Controls					
15-2	0.11	0.23	5.4	3.9	19
16-8	0.08	0.28	5.3	3.8	22
13-5	0.06	0.26	5.5	4.0	20
18-9	0.06	0.25	5.4	3.9	23
17-5	0.05	0.17	5.4	3.9	20
Mean[a]	0.07±0.03	0.24±0.03	5.4±0.1	3.9±0.1	20.8±1.0

[a]Expressed as mean ± SEM

Appendix 10. Study I, Group 2 - Clinical Chemistry Values 4 Weeks Post-Transplantation

Animal Number	ALT IU/L	APT IU/L	SDH IU/L	Alk. Phos. IU/L	GGT IU/L	LAP IU/L	LDH IU/L
Tumored							
5-1	32	56	6	243	8	27	174
1-4	74	81	9	185	3	24	410
2-6	115	119	14	201	6	25	334
9-2	67	86	10	263	5	25	476
12-10	65	77	10	176	9	18	279
7-2	47	66	7	263	11	27	182
3-2	58	78	9	236	6	25	165
10-6	86	83	14	270	9	27	132
11-3	95	101	10	267	9	27	337
6-8	89	89	9	221	6	29	132
Mean[a]	72.8±6.4	83.6±4.7	9.8±0.9	232.5±10.3	7.2±0.7	25.4±0.8	262±33.0
Controls							
16-10	19	54	14	311	0	25	151
14-7	15	44	11	308	2	27	77
13-1	18	33	10	297	5	27	96
18-8	12	44	6	251	3	24	69
17-4	16	50	7	269	2	25	61
Mean[a]	16.0±9.1	49.0±6.6	9.6±1.2	287.2±14.6	2.4±1.0	25.6±1.2	90.8±46.6

[a]Expressed as mean ± SEM

Appendix 10. Continued

Animal Number	D. Bili. mg%	T. Bili. mg%	T. Prot. gm%	Alb. gm%	BUN mg%
Tumored					
5-1	0.02	0.17	5.4	3.7	23
1-4	0.00	0.22	5.5	3.5	23
2-6	0.02	-	5.4	3.5	29
9-2	0.03	-	5.3	3.5	29
12-10	0.02	0.11	5.2	3.2	24
7-2	0.00	0.09	5.9	3.9	25
3-2	0.02	0.19	5.7	3.8	28
10-6	0.00	0.20	5.7	3.4	30
11-3	0.02	0.25	5.8	3.6	37
6-8	0.02	0.20	5.6	3.4	27
Mean[a]	0.02±0.01	0.18±0.02	5.3±0.1	3.7±0.0	21.2±0.7
Controls					
16-10	0.08	-	5.8	4.4	28
14-7	0.00	0.22	5.9	4.4	25
13-1	0.00	0.30	5.7	4.3	24
18-8	0.00	0.17	5.6	3.4	26
17-4	0.03	0.19	5.7	4.0	27
Mean[a]	0.02±0.01	0.22±0.03	5.4±0.2	3.9±0.1	20.8±1.0

[a]Expressed as mean ± SEM

Appendix 11. Study I, Group 3 - Clinical Chemistry Values 6 Weeks Post-transplantation

Animal Number	ALT IU/L	APT IU/L	SDH IU/L	Alk. Phos. IU/L	GGT IU/L	LAP IU/L	LDH IU/L
Tumored							
6-7	215	191	10	105	9	25	501
2-9	88	103	4	179	6	22	642
8-8	181	223	7	86	8	27	760
12-7	360	235	23	89	6	24	498
8-6	348	369	12	95	11	25	495
1-1	233	186	11	78	12	25	549
11-1	98	94	7	122	5	22	482
7-3	196	226	9	78	6	24	544
4-8	106	102	6	200	6	25	279
12-8	255	174	12	110	11	24	452
Mean[a]	208.0±25.3	190.2±21.7	10.1±1.5	114.2±12.6	8.0±0.7	24.3±0.6	520.2±36.3
Controls							
16-7	14	47	3	209	6	22	260
18-6	16	53	10	234	3	22	225
18-7	12	56	6	291	3	24	342
16-9	14	54	4	230	5	24	222
17-3	14	73	6	212	3	18	430
Mean[a]	14.0±35.8	56.6±30.8	5.8±2.1	235.2±17.8	4.0±1.0	22.0±0.8	295.8±51.4

[a]Expressed as mean ± SEM

Appendix 11. Continued

Animal Number	D. Bili. mg%	T. Bili. mg%	T. Prot. gm%	Alb. gm%	BUN mg%
Tumored					
6-7	0.08	-	5.9	4.1	20
2-9	0.13	-	5.6	4.1	24
8-8	0.06	0.23	5.3	3.9	25
12-7	0.02	0.28	5.7	4.0	27
8-6	0.11	0.34	5.8	4.4	24
1-1	0.06	0.28	5.7	4.0	30
11-1	0.06	-	5.6	3.7	20
7-3	0.05	-	5.6	4.3	31
4-8	0.03	-	5.8	4.2	22
12-8	0.09	6	5.7	4.0	18
Mean[a]	0.07±0.01	-	5.7±0.0	4.1±0.1	24.1±1.3
Controls					
16-7	0.06	-	5.7	4.4	17
18-6	0.05	-	5.7	4.0	19
18-7	0.05	-	5.9	4.6	25
16-9	0.11	-	6.1	4.5	21
17-3	-	-	-	-	-
Mean[a]	0.07±0.0	-	5.8±0.1	4.4±0.1	20.5±2.0

[a]Expressed as mean ± SEM

Appendix 12. Mammary Homogenates

Purpose

The R3230AC mammary adenocarcinoma in the Fischer 344 rat is considered comparable to the mammary gland of a rat during late gestation and early lactation (Hilf 1967, McGuire 1969, Witliff et al 1972, Klein and Loizzi 1977). This study was designed to determine if the normal mammary gland during late gestation and early lactation produced or contained alanine aminotransferase. The presence of high enzyme activities within the mammary gland might help explain its presence in the R3230AC-L tumor.

Materials and Methods

Ten pregnant female Fischer 344 rats within 5 days of their casting dates were weighed and then sedated with carbon dioxide vapor in the form of dry ice.[a] A blood sample was obtained from each rat via cardiac puncture. The rat was killed by exsanguination. Part of the blood sample was placed in a vial containing ethylenediamine tetraacetic acid[b] for hemoglobin determination with an automated electronic analyzer.[c] The rest of the sample was placed in a silicone coated glass vial[b] and allowed to clot. The serum was aspirated from the clot after

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[a]Cardox®, Division of Chemetron Corporation, Countryside, Illinois.

[b]Vacutainer, Becton, Dickinson and Company, Rutherford, New Jersey

[c]Coutler S., Coulter Electronics, Inc., Hialeah, Florida

centrifugation at 2000 revolutions per minute for 15 minutes. The alanine aminotransferase activity of the serum sample was determined with an automated centrifical analyzer.[d]

The mammary glands were disected from the subcutaneous tissue, weighed and placed in 0.15 molar sodium chloride. The samples were maintained in an ice bath at all times to prevent enzymatic degredation. The anterior group of mammary glands were placed in a separate container from the posterior group. Using more than one gland, several small pieces of mammary gland were collected until the combined weight was approximately 150 mg from each group (i.e., the anterior and posterior groups of mammary gland). These pieces were placed in a 10 ml volumetric flask and cold 0.15 molar sodium chloride was added to a total volume of 10 ml. The tissues were initially minced by hand. The total volume was then brought up to 30 ml with additional cold saline. The tissues were further homogenized in a Potter-Elvehjen glass homogenizer[e] fitted with a motor driven[f] teflon pestle. The mammary gland sample from each rat was homogenized for 2 one-minute periods at 1000 revolutions per minute according to the method of Schneider (1961). The samples were transferred to centrifuge tubes and were spun in a pre-cooled refrigerated centrifuge (2-4°C)[g] for 15 minutes at 1500 x g. The supernatant was decanted and subjected to centrifugation in a high speed refrigerated centrifuge (2-4°C)[h] at 39,300 x g for one hour. The water clear supernatant was pipetted into glass vials which were sealed

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[d]Centrificheim, Union Carbide Corp., Rye, New York

[e]A. H. Thomas Company, Philadelphia, Pennsylvania

[f]Tri-R Stir-R Model K41, Tri-R Laboratory Instruments, Inc., New York, New York

and stored in a refrigerator (5-10°C) until enzymatic analysis could be done the next day.

The tissue analyses for alanine aminotransferase and hemoglobin were done using an automated centrifical analyzer.[i] A control serum sample[j] was also analyzed as an external control of consistancy in the results. The method for alanine aminotransferase levels was according to Wroblewski and LaDue (1956). The method for hemoglobin in the tissues was according to Standefer and Vanderjagt (1977). The hemoglobin values were used to factor out that part of the tissue alanine aminotransferase which was due to blood contamination.

Results and Discussion

The alanine aminotransferase level in the mammary tissue was very low (Table 15). The mean activity, expressed as international units per gram of tissue, was 0.51 ± 0.24. This would suggest that the R3230AC-L tumor does not contain alanine aminotransferase activity by virtue of its similarity to normal mammary tissue of rats during late gestation and early lactation.

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[g]Beckman Model TJ-6, Beckman Instruments, Inc., Palo Alto, California.

[h]Sorvall Superspeed, Model RC2-B, Dupont Instruments, Biomedical Division, Newtown, Connecticut.

[i]Centrifichem, Union Carbide Corporation, Rye, New York.

[j]Moni-Trol I, Dade Division, American Hospital Supply Corporation, Miami, Florida.

Table 15. ALT activity in serum and mammary tissues from rats during late gestation

Animal Number	Serum (IU/l)	Mammary Gland (IU/gm)
1	14	0.4
2	16	0.3
3	24	0.7
4	17	0.5
5	14	0.6
6	14	0.8
7	16	0.5
8	19	0.5
9	16	0.8
10	28	0.9

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Appendix 13. Lung Homogenates

Purpose

When the R3230AC-L tumor is transplanted beneath the kidney capsule in female Fischer 344 rats, there is a gradual increase in serum levels of alanine aminotransferase as the tumor grows and as it metastasizes to the lung. This is not statistically significant until the tumor has metastasized. This study was designed to determine if the lung was responsible in part for the elevated alanine aminotransferase observed in the serum.

Materials and Methods

Ten female Fischer 344 rats four weeks after renal transplantation of the R3230AC-L tumor were utilized. Ten rats which had been sham operated at the same time served as controls.

For ease of handling, each rat was sedated with the carbon dioxide vapor from dry ice.[a] A blood sample was obtained via cardiac puncture. The rat was killed by exsanguination. Part of the blood sample was placed in a vial containing ethylenediamine tetraacetic acid[b] for hemoglobin determination with an automated electronic analyzer.[c] The remainder of the sample was placed in a silicone coated glass vial[b] and

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[a]Cardox®, Division of Chemetron Corporation, Countryside, Illinois

[b]Vacutainer, Becton, Dickinson and Company, Rutherford, New Jersey.

[c]Coulter S., Coulter Electronics, Inc., Hialeah, Florida.

allowed to clot. The serum was separated from the clot after centrifugation at 2000 revolutions per minute for fifteen minutes. The alanine aminotransferase activity of the serum was determined with an automated centrifical analyzer.[d]

The lungs were removed, weighed and placed in 0.15 molar sodium chloride. The samples were maintained in an ice bath at all times to prevent enzymatic degredation. Separate samples were taken from lungs of the tumor-bearing rats so that unaffected lung tissue and metastases could be analyzed separately from these rats. At this stage of the tumor development the metastases were frequently quite small and it was difficult to obtain large samples. The samples for homogenization were weighed. Those weighing 60 milligrams or less were placed in a 2 ml volumetric flask. Those weighing more than 60 mg were placed in a 10 ml volumetric flask. Sufficiently large samples could be obtained from the control rats' lungs so that these were always placed in the 10 ml volumetric flasks, (Tissue samples from the control rats weighed within the range of 100 $\pm$ 25 mg).

The flasks were brought up to volume with cold 0.15 molar sodium chloride. The tissues were then homogenized in an appropriate size Potter-Elvehjen glass homogenizer[e] fitted with a motor driver[f] teflon pestle. Each sample was homogenized for 2 one-minute periods at 1000 revolutions per minute according to the method of Schneider (1961). Next they were transferred to centrifuge tubes and were spun in a

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[d]Centrifichem, Union Carbide, Corp., Rye, New York.

[e]A. H. Thomas Company, Philadelphia, Pennsylvania.

[f]Tri-R Stir-R Model K41, Tri-R Laboratory Instruments, Inc., New York, New York.

pre-cooled refrigerated centrifuge (2-4°C)[g] for 15 minutes at 1500 x g. The supernatant was decanted and centrifuged in a high speed refrigerated centrifuge (2-4°C)[h] at 39,300 x g for one hour. This supernatant was pipetted into glass vials. The vials were sealed and were stored under refrigeration (5-10°C) until enzymatic analysis could be done the next day.

The tissue analyses for alanine aminotransferase and hemoglobin were performed with an automated centrifical analyzer.[i] A control serum sample[j] was also analyzed as an external control of consistency of the results. The method for alanine aminotransferase levels was according to Wrobleski and LaDue (1956). The method for hemoglobin in the tissues was according to Standefer and VanDerjagt (1977). The hemoglobin values were obtained for factoring out that part of the tissue alanine aminotransferase which was due to blood contamination.

Results and Discussion

The mean activity of alanine aminotransferase remained very low in both the normal lung tissue of the tumor-bearing rats and the lung tissue of the sham operated rats with mean values of 1.1 ± 0.2 IU/gm and 1.8 ± 0.3 IU/gm respectively (Table 16). The metastases level of alanine aminotransferase averaged 3.4 times the level of the enzyme in

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[g]Beckman Model TJ-6, Beckman Instruments, Inc., Palo Alto, California.

[h]Sorvall Superspeed, Model RC2-B, Dupont Instruments, Biomedical Division, Newtown, Connecticut.

[i]Centrifichem, Union Carbide Corporation, Rye, New York

[j]Moni-Trol I, Dade Division, American Hospital Supply Corporation, Miami, Florida

the "normal" lung tissue from the same animal. It would appear that the metastatic tumor tissue and not the lung contributed to the elevation of serum levels of alanine aminotransferase observed in rats bearing the R3230AC-L tumor.

Table 16. Serum, lung and metastases activity of alanine aminotransferase

Tumor-bearing Rats				Control Rats		
Animal Number	Serum IU/L	Lung IU/gm	Metastases IU/gm	Animal Number	Serum IU/L	Lung IU/gm
1	44	1.2	3.4	1C	19	2.0
2	49	0.8	2.1	2C	16	2.3
3	60	0.8	2.3	3C	19	1.7
4	37	1.1	3.2	4C	23	1.7
5	32	1.4	a	5C	19	1.8
6	28	1.4	3.5	6C	17	1.7
7	145	0.7	4.9	7C	23	1.9
8	94	1.1	5.2	8C	17	1.8
9	71	1.2	2.6	9C	16	1.4
10	85	1.1	3.3	10C	16	2.2
Mean[b]	64.5±36.0	1.1±0.2	3.4±1.1		18.5±2.7	1.8±0.3

[a]No metastases observed grossly.

[b]Expressed as mean ± SEM.

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VITA

The author was born Christine Ratke on September 25, 1946 in Detroit, Michigan. She received her Bachelor of Science degree in 1965 and her Doctor of Veterinary Medicine degree in 1969 from Michigan State University. Her academic honors included: membership in Phi Kappa Phi and Phi Zeta, both degrees conferred "with high honor," an award for proficiency in small animal medicine and an award for highest grade point average in her veterinary school graduating class. After graduation, she became a resident in veterinary radiology and assisted teaching veterinary students at the Michigan State University Veterinary Clinic for 1-1/2 years. She married Alexander Dale Hall, D.V.M. in 1971. From 1971 to 1976 she was a small animal private practitioner in Massachusetts. In 1976, she and her husband returned to Michigan State University to obtain advanced degrees in veterinary pathology. As part of her training, she worked as a graduate assistant in veterinary clinical pathology. In 1977 she received The Upjohn Post-Doctoral Fellowship. In 1979 she was employed as a temporary veterinary pathologist at The Upjohn Company as part of a cooperative educational program between Michigan State University and The Upjohn Company. It was through this program that most of her research support came.