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Radioiodinated Peanut Lectin and Monoclonal Antibodies for the detection of T-Antigen expressing Tumors and Metastases

by

Soon Ming Eu

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Pharmaceutical Sciences (Radiopharmacy)

Faculty of Pharmacy and Pharmaceutical Sciences

EDMONTON, ALBERTA

FALL 1985

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Abstract

The peanut lectin (PNA) has high preferential binding affinity for oligosaccharides containing the terminal sequence βDGal(1→3)GalNAc. This disaccharide is also the immunodominant structure on the Thomsen-Friedenreich (T) antigen which is present in a non-cryptic manner on a number of human and animal adenocarcinomas but not on corresponding normal or benign tissues. Radioiodinated peanut lectin was investigated as a potential radiopharmaceutical in the detection of T antigen expressing solid tumors and metastatic lesions using the T antigen expressing TA3/Ha mammary adenocarcinoma and its selected organ specific metastatic variants as the animal tumor model.

The peanut lectin was labeled with I-125 using the iodogen method resulting in a radiopharmaceutical that retained its immunological activity and radiochemical stability for at least a week when stored at 4° C. Radioiodinated peanut lectin was found to bind avidly and specifically *in vitro* to various T antigen expressing animal tumor lines and neuraminidase treated red blood cells. Tissue biodistribution studies with ^{125}I-PNA in the TA3/Ha tumor bearing mice revealed high retention of PNA by the tumor mass while other organs demonstrated a rapid clearance of the protein. The tubules of the kidneys are suspected of being involved in a unique mechanism of binding and excreting the peanut lectin when it was found that PNA was excreted in an active form in the urine. Clear scintigraphic delineation of the tumor was seen by 24 and 48 hours post-injection without the need for body background subtraction. Biodistribution studies revealed high tumor:blood ratios of 7:1 and 55:1 at 24 and 48 hours with corresponding tumor:muscle ratios of 33:1 and 77:1 at the same time periods. The tumor uptake of radioactivity due to metabolically released I^- from I-125 PNA was minimal as there was only 0.3% of the injected $Na^{125}I$ dose per gram of tumor tissue at 24 hours post-injection. Localization index values calculated for ^{125}I-PNA localization at the tumor site using ^{131}I-F(ab')$_2$ fragments of IgG as the nonspecific protein indicated that the PNA localization at the tumor site was highly specific. It was found that ^{125}I-PNA administered in the dose range of 0.1 μg to 1.0 μg per 20 g mouse gave the highest tumor

localization index while uptake of PNA by the other organs were minimal.

Tissue biodistribution studies of mice bearing the organ specific TA3/Ha metastatic tumor variants revealed that the lungs or liver containing the tumor lesions retained about 7 to 8 times as much of an intravenous ^{125}I-PNA dose as did the controls, thereby allowing for clear delineation of the tumor infiltrated lungs or liver by gamma scintigraphy. Autoradiograms of the liver tissue sections containing the TA3/Ha metastatic variant lesions after ^{125}I-PNA intravenous administration demonstrated avid uptake of PNA throughout the viable tumor mass and FITC-PNA staining of these tissue sections readily identified the metastatic lesions under fluorescence microscopy.

The data obtained from all these studies illustrate the potential usefulness of radiolabeled peanut lectin for the detection and delineation of T antigen expressing solid tumors and metastatic lesions.

An IgM monoclonal antibody that has high affinity for the high molecular weight glycoprotein, epiglycanin, present on the surface of the TA3/Ha cells was investigated as a potential tumor radioimmunoimaging agent. *In vitro* studies demonstrated the affinity of anti-epiglycanin for TA3/Ha tumor cells and neuraminidase treated red blood cells. T antigenic specificity was not demonstrated for anti-epiglycanin as the presence of PNA did not affect the binding activity of anti-epiglycanin *in vitro* as well as *in vivo*. Gamma scintigraphic imaging of ^{125}I anti-epiglycanin in TA3/Ha tumor bearing mice demonstrated intense localization of radioactivity at the tumor site but tissue biodistribution studies found that anti-epiglycanin did not accumulate much more than the nonspecific IgM proteins at the tumor site. Further studies with the IgG class or its F(ab')$_2$ fragments of the anti-epiglycanin monoclonal antibody may reveal the potential utility of this line of monoclonal antibodies as tumor radioimmunoimaging agents.

Acknowledgements

I wish to express my sincere gratitude to Dr. Alec Shysh for his support and guidance throughout the course of the research and in the preparation of this manuscript, without whose help and [illegible] this work would not have been possible.

I wish to thank Drs. A.A. [illegible] M.R. Suresh for their expert advice and consultation; Dr. B.M. Longenecker and his staff at the Department of Immunology, University of Alberta, for their help and supply of antibodies and tumor cell lines; Connie Turner and Lindsay J. MacQueen for their help with the animal work; Chris Ediss and Steve McQuarrie for their technical help and mathematical consultation; and Dr. Yip W. Lee, Thomas Woo, Graeme R. Boniface, Ingrid Koslowsky, Brad Hunter and Dr. S. Selvaraj for their ever-willing helping hand.

To my parents, I owe a special kind of debt and thanks for their understanding and love.

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I. Introduction

The concept of tumor detection by utilizing a radiolabeled tracer that has some degree of specific localization in malignant neoplasms at concentrations sufficient to detect and delineate reliably and accurately the spread of both the primary tumor and distant metastases has been considered for a long time. An approach to this concept is to utilize radiolabeled tracers such as lectins and antibodies that selectively bind to specific antigens on tumor cell membrane surfaces. Tumor associated antigens could arise when normal cells that undergo malignant transformation modify their surface properties such as altering their membrane-bound glycoproteins and glycolipids due to incomplete and/or abnormal synthesis of these molecules.

The Thomsen-Friedenreich (T) antigen which has been reported to occur in a non-cryptic form on many human adenocarcinomas but not on other diseased or healthy tissues has been commonly referred to as a tumor associated antigen. This same T antigen has also been indicated as having a role in the process of cancer invasion and metastasis. The peanut lectin which has an avid and specific binding affinity for the immunodominant carbohydrate structure of the T antigen is investigated here as a potential radioimaging agent of T antigen expressing solid and metastatic tumors using the TA3/Ha mammary adenocarcinoma and its selected organ specific metastatic variants.

The TA3/Ha tumor model, a spontaneous mammary adenocarcinoma of strain A mice, resembles the human adenocarcinoma in its spontaneous origin and its content of human blood-group like antigen specific structures. These features along with the availability of the organ specific metastatic variants provides an animal tumor model for the evaluation of various other macromolecules such as monoclonal antibodies for the radioimmunodetection of solid tumors and metastatic lesions.

An IgM monoclonal antibody that has binding affinities for the TA3/Ha tumor cell surface glycoprotein, epiglycanin, is also investigated for its potential as a radioimmunoimaging agent. It is, therefore, the aim of this project to evaluate the potential of radiolabeled peanut lectin and anti-epiglycanin IgM monoclonal antibody as diagnostic radiopharmaceuticals in the

detection of selected primary and metastatic tumors, and also to evaluate the TA3/Ha mammary adenocarcinoma and its organ specific metastatic variants as appropriate animal tumor models.

II. Survey of the literature

A. Tumor detection

The use of radiopharmaceuticals in the detection and diagnosis of tumors provides a non-invasive method that allows the clinician to determine the location of neoplasms and any distant metastases, their extent and the evaluation and follow up of treatment[1]. Radiodiagnosis and detection of tumor tissues is only possible when there is a preferential uptake of the administered radiopharmaceutical by the tumor cells in sufficient concentrations to enable a clinically useful scan[1,2]. It has been determined that a tumor to background tissue ratio of at least 2:1 is needed to detect tumor lesions of about 2 centimeter in diameter[2] and hence, smaller lesions might be detected when there is greater concentration and a higher tumor to background tissue ratio of an administered radiopharmaceutical in a tumor.

Radiopharmaceuticals used for the diagnostic imaging of tumors can generally be categorized into three groups; i) labeled compounds and radionuclides, ii) labeled antibiotics and iii) labeled antibodies, and furthermore, the method of localization of the radiopharmaceuticals can be divided into two such as direct and indirect[1].

Indirect methods of tumor localization

Indirect methods of tumor imaging depend largely upon the alterations of normal tissue function and structure caused by the presence of a tumor process in that tissue which would lead to an abnormal uptake of an administered radiopharmaceutical resulting in an abnormal scan[1]. Most of the labeled compounds and radionuclides employed fall into this category of tumor localization and these compounds are also often called "non-specific" tumor localizing agents. Such agents are usually non-specific in their tumor localization as they also localize in other non-malignant and non-specific processes such as inflammation and certain phases of infarcts[3]. Another drawback to this method is that usually only one radiopharmaceutical is useful for one type of tissue and thus, different radiopharmaceuticals need to be administered

for the evaluation of different organ systems[3].

Examples of commonly used radiopharmaceuticals in this category are i) Tc-99m sodium pertechnetate which upon administration gives a "hot" spot in areas of the brain where an area of increased permeability of the blood brain barrier is present, ii) radiolabeled colloid which gives a "cold" area in the liver due to lack of uptake because of parenchymal destruction, and iii) Tc-99m phosphates which in increased osteoblastic activity in bone give a "hot" area[3].

Direct methods of tumor localization

Direct methods of tumor localization by radiopharmaceutical agents rely upon the preferential uptake of the agent by the tumor. The radiopharmaceuticals in this catgory are usually termed "specific" agents as the mechanism of localization is rather specific for the tumor resulting in an increased uptake by the tumor such as:

1) increased metabolism with resulting increased uptake of radiolabeled substrates such as ^{131}I-iodocholesterol and ^{75}Se-selenomethionine[4],

2) increased affinity of certain agents for tissues such as Gallium-67 citrate in soft tissue tumors[5,6],

3) antibiotics with selective uptake and action on tumors such as radiolabeled bleomycin or adriamycin[1], and

4) antigen-antibody reaction with radiolabeled antibodies raised against tumor associated antigens[1].

Radiolabeled substrates such as I-131 iodocholesterol, Se-75 selenomethionine and other radiolabeled amino acid, steroids or sugar analogs have been used to exploit the increased rates of protein synthesis as an accumulating factor in tumor processes[1]. Detection of the tumor depends upon the accelerated intermediary metabolism of tumors compared to the normal parent tissue from which the tumor arose. Tumor imaging can hence be done with tailored metabolites to particular tumors. Se-75 Selenomethionine, a labeled essential amino acid, would be taken up by tumors with an increased rate of protein synthesis and this

compound has been used in the diagnosis of hepatomas and lymphomas[4]. Iodine-131 labeled iodocholesterol has been used to accumulate in cortisol secreting adenomas and iodine-131 sodium iodide solution which is taken up avidly by hyperactive thyroid metastases results in a "hot" area compared to normal parent tissue[1]. However, these agents do suffer from the drawback that they are unable to interact with a wide variety of tumors and, as such their routine use in diagnostic imaging has diminished considerably giving way to tumor imaging agents with broader spectra of activity[4].

The potential utility of Gallium-67 citrate as a tumor imaging radiopharmaceutical was first suggested in 1969 following the serendipitous discovery of the localization of this agent in tumors by Edward and Hayes[11]. Since then, Gallium-67 has been investigated in a variety of tumors to evaluate its potential as a tumor localizing agent and it is probably the most frequently used tumor imaging agent at present. Gallium-67 citrate, a radiopharmaceutical found to have increased affinity for tumors, has been reported to be useful in the detection of specific tumors and many comprehensive reviews of these reports have been published in the literature. Gallium-67 scanning has been found to be useful for the entire process of evaluating patients with lymphomas (Hodgkin's disease and malignant lymphomas) including the initial staging and evaluation of the response to therapy[5,6]. Gallium-67 radiolocalization scintigraphy is also being increasingly used as a means of defining the extent of lung carcinomas although differences in ^{67}Ga uptake exist for the various tumor types with anaplastic small cell carcinoma demonstrating the overall highest uptake while adenocarcinoma and anaplastic large cell carcinoma having the lowest[5]. Gallium-67 scintigraphy is used in the detection of liver metastases and hepatomas especially in patients with cirrhosis[5]. Since a large fraction of the injected dose is concentrated by the hepatocytes a gallium-67 liver scan alone cannot be adequately used to assess the liver without an accompanying liver scan with radiolabeled sulfur colloid[5]. The sensitivity of the gallium scan in the detection of gastrointestinal tract and related organ malignancies, genitourinary tract tumors and its metastases, and testicular malignancies has been found to be rather low[5]. Gallium-67 citrate has also been found not to be superior

over some other available radiopharmaceuticals or procedures for the detection of some tumors such as bone metastases[5]. Gallium-67 was also reported not useful for the diagnostic evaluation of myeloma patients or in the early detection and staging of carcinoma of the breast[5]. Other indications for the use of Gallium-67 citrate are as listed below;

1) establishing the extent of primary tumors, especially for tumors that are situated in the chest,

2) detecting any additional tumor foci, especially previously unknown metastases,

3) therapy planning such as in Hodgkin's disease and other malignant lymphomas,

4) differentiating diagnostically between cerebrovascular lesions and brain tumors,

5) indicating any residual tumor or recurrence of tumor after therapy, and

6) evaluating treatment efficacy and tumor susceptibility to irradiation[5,6].

The diagnostic value of Gallium-67 citrate as a tumor imaging agent has been limited by the nonspecific uptake of Gallium-67 in non-neoplastic tissues as well as in various inflammatory diseases including abcesses and benign granulomas particularly sarcodosis. The sensitivity of tumor imaging using Gallium-67 has been shown to depend on the percentage uptake, the tumor to background tissue ratio, tumor size and type, and the viability of the tumor cells[5,6,11].

The use of antibiotics with selective action on tumors as tumor imaging agents has been demonstrated with various radiolabeled antibiotics such as bleomycin, adriamycin, tetracycline, and streptozotocin[1]. The forerunner in the use of antibiotics as tumor detecting agents was bleomycin. Bleomycin consists of a group of antibiotics with demonstrated antineoplastic activities against a variety of different tumors and may have a useful role in the detection of tumors and staging, and in the differential diagnosis of certain types of lesions[12], as well as antimicrobial activity but was unfortunately found to be toxic in therapeutic doses. Bleomycin can be labeled with a variety of radionuclides such as technetium-99m, gallium-67, cobalt-57, indium-111, iodine-123, platinum-195m and gold-198 without loss in activity[12]. However, the label on the radiolabeled bleomycin was found to be unstable *in vivo* often resulting in the

release of the free radioactive label into the circulation soon after administration[11,12]. Furthermore, bleomycin was found not to be tumor specific as it was seen to localize in non-malignant processes such as infections[1]. The use of other radiolabeled antibiotics in tumor imaging has diminished as these agents were found not to be as tumor specific as had been hoped and tumor to background tissue ratios obtained were found to be rather low [11].

The use of rabiolabeled antibodies for the diagnostic detection of tumors was first demonstrated by Pressman in 1957[17]. Since then, there have been numerous reports by various investigators[13] of the use of radiolabeled antibodies for the detection of tumors and some of the potential clinical applications of radiolabeled antibodies in diagnostic tumor imaging are listed as[8]

1) the detection,location and determination of the extent of the tumor,

2) the characterization of the neoplasm cell type,

3) the follow up of progress of residual lesions left after initial treatment,

4) the early identification of recurrence of metastasis and

5) the assesment of treatment efficacy.

The above mentioned potential uses are what an investigator hopes to achieve with radiolabeled antibodies but unfortunately, most researchers have met with difficulties in the development of specific antibodies to tumor antigens and hence the ability to specifically detect tumors was not generally available. In the development of radiolabeled antibodies to tumors, there are a number of factors that have to be considered including[14-16]

1) the isolation of tumor associated antigens that can offer the most specificity to the tumor for the production of antibodies. Some clinical trials have demonstrated that even elaborate purification of antigens have not resulted in an absolutely specific antibody as evidenced by the cross reactivity with other tumors and normal tissues,

2) the selection of cellularly localized antigens that permit the greatest access to the antibody interaction. The location of physical contact between antigen and antibody should be extravascular and fortunately, much of the neoplasm vasculature does not

have competent walls such as those found in normal capillaries which then would allow the escape of circulating antibody into the interstitial fluid. There have been reports of tissue antigens that are not readily accessible to the interstitial fluid and antibody[14]. Cellular surface antigens or antigen shed into the interstitial space of the tumor presents the most advantageous physical situation for efficient antigen-antibody reaction whereas intracellular antigens offer the least promising state as it is difficult for antibodies to gain access into the cellular cytoplasm,

3) the selection of antigens that will give high titer antibodies,

4) the selection against cross-reacting antibodies,

5) the radionuclide labeling of stable antibodies with high enough specific radioactivity to allow useful studies of the antibody without loss of immunoreactivity,

6) the ability to produce antibody fragments without undue loss of immunoreactivity and

7) the production of antibodies with sufficient *in vivo* stability.

In earlier studies, the lack of tumor specificity associated with the of radiolabeled antibodies was attributed to the difficulties in isolating and defining a tumor specific or tumor associated antigen[14] and in obtaining well-defined and reproducible antibodies.

With the advent of monoclonal antibodies[9], many of the problems such as antigen specificity and the reproduction and definition of antibodies encountered with the use of polyclonal antisera were eased. This technique developed by Kohler and Milstein[9] made possible the production of large quantities of antibodies of pre-defined specificity with a monospecific antigen reactivity.

Tumor associated markers

Neoplastic transformation of normal tissue is often accompanied by the acquisition of new and specific antigenic moieties which are not usually detected in normal and untransformed tissue[10,14]. Hence, antibodies can be raised against these tumor associated antigens found on

tumors to produce potentially useful specific antibodies for tumor localization and other clinical applications[10]. Most tumor associated antigens that have been categorized biochemically on the basis of their composition and structure are found to be glycoproteins although there is evidence suggesting that other types of molecules could also be potential cell surface tumor markers[16]. Accumulating evidence has shown that human tumor tissues synthesize, express, and sometimes even release into circulation tumor specific antigenic substances which are either absent from or produced in very limited quantities by the original parent tissue[10]. Ideally, tumor associated antigens should be expressed on the surface of the cancer cells and be immunologically detectable, thus making them available for interaction with rabiolabeled antibodies *in vivo*[16]. It has been deemed highly desirable to have a tumor associated antigen that is a specific and sensitive indicator of malignancy with any shedding of the antigen into the urine or serum in proportion to the degree of the tumor burden. Other potentially ideal charactersitics of tumor associated antigens are that they should be expressed during all stages of the cell cycle with relatively uniform distribution on primary lesions and metastases, and upon binding with a radiolabeled antibody the complex should not be shed rapidly but allow a long enough residence time at the tumor site to enable detection[16]. There are several categories of tumor associated antigens recognized in human neoplasms such as[10]:

1) oncofetal antigens including carcinoembryonic antigen and alphafetoprotein,

2) placental antigens such as human chorionic gonadotrophin and human placental antigen found normally in the serum of pregnant women,

3) protein and polypeptide hormones which have been found to be secreted by many human tumors even though the normal parent tissue may not synthesize the particular hormone,

4) exocrine products which are normally secreted only during pregnancy and lactation that have been found in some cases of breast cancer, and

5) products of altered cell surface glycosylation which may have resulted from the altered activity of one or more glycosyltransferases that may lead to changes in the

biosynthesis of cell surface glycolipids and glycoproteins. One such example is the expression of the Thomsen-Friedenreich antigen that normally occurs in a cryptic form in normal cells.

B. Thomsen-Friedenreich antigen

The Thomsen-Friedenreich antigen (T-antigen) was originally described as a rare erythrocyte antigen in 1928 when it was discovered that the presence of bacterial contamination uncovered a normally hidden receptor on erythrocytes which rendered the blood agglutinable by all human sera including that of the donor[18]. The cause of the Thomsen-Freidenreich phenomena was found to be due to an enzyme present in certain bacteria and viruses[19]. This Receptor Destroying Enzyme (RDE) or sialidase[20], now called neuraminidase, causes the release of terminal sialic acid, N-acetyl-neuraminic acid (NAN), and unmasks a specific haptenic structure present on all human and many animal erythrocytes with the exception of human infant erythrocytes[18] or on NAN depleted circulating senescent red blood cells[21]. It has been further demonstrated that the Thomsen-Friedenreich transformation not only occurs on red blood cells from humans and several animal species but also on liver, muscle and most strongly on brain tissue[22].

Physical and chemical composition of the Thomsen-Friedenreich antigen

The physical and chemical characteristics of the Thomsen-Friedenreich antigen (T-antigen) are quite well documented. Treatment of the MN erythrocyte glycoproteins on red blood cells with neuraminidase and mild acid hydrolysis results in the exposure of the T antigen with the concomitant release of neuraminic acid[23,24]. The T antigen then isolated from human erythrocytes was found to be an aggregate of repeating apparently identical subunits[22]. The physical characteristics of the Thomsen-Friedenreich antigen on human red blood cells were determined as[22]:

Sedimentation constant	11.1 s

Diffusion constant	1.62 F
Partial specific volume	0.696 mL/g
Molecular weight	555 000 daltons

The chemical composition of the T antigen was found to be approximately 40% carbohydrate of which galactose was 13.2%, N-acetylgalactosamine was 10.3%, N-acetylglucosamine was 7.4%, mannose was 6.4% and fucose was 0.83%. The remainder is protein which was found to be rich in threonine, serine, leucine and glutamic acid while there was no cysteine or tryptophan found[24-26].

The Thomsen-Friedenreich receptor molecule has been defined as belonging to the glycophorin type of erythrocyte membrane glycoprotein located near or on the $-NH_2$ terminal area[27], with the immunodominant T specific structure βDgalactosido (1→3)αNacetylD-galactosamine linked α,O-glycosidically to serine or threonine on erythrocyte derived glycoproteins[24]. The normally cryptic T structure is a protein bound and an alkali labile disaccharide which upon desialylation represents the carbohydrate part of the MN blood group antigens[28]. Accordingly, the T receptor has been determined to consist of two parts; one is the immunodominant carbohydrate structure chain in the form of the T disaccharide that is common to all T receptors and secondly, the NH_2- terminal protein part of the glycophorin type membrane glycoproteins that may vary from species to species and even from M to N specificity[28].

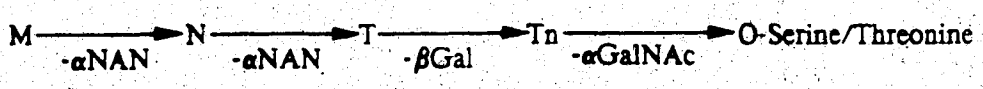
M-, N-, T-, Tn-, blood group specificities

The blood group M and N antigens are the major antigens of the second human blood group system. Human red blood cell M and N antigens are aggregates of sialoglycoproteins, evidently composed of identical subunits[29], having molecular weights ranging from $3x10^4$ to at least $1.2x10^7$ depending upon the method of isolation[26]. The overall physical and chemical composition of the M and N antigens are basically similar[26] but differ in terminal immunodominant carbohydrate structures which clearly distinguish them from one

another[23,24,29]. The peptides that carry the M and N specific structures on erythrocytes differ from one another in two amino acids; serine being the only NH_2 terminus for M and leucine being the only one for N, and the amino acid fifth from the NH_2 terminus is glycine for M and glutamic acid for N[22]. It has been reported that nearly 85% of the carbohydrate chain of the MN glycoproteins are linked via GalNAc to threonine or serine and are alkali labile[24]. N-acetylneuraminic acid was determined to be an essential component of both M and N specificities[23,29].

The immediate biosynthetic precursor of the MN blood group system are the T antigen and the Tn antigen[23,24,30]. However, it was reported that covered T and Tn specific structures also do occur in carbohydrates that do not terminate in the M or N immunodeterminants[21,25], such as the observation of T specific structures in epithelial tissues including salivary and breast glands and kidneys[33]. The T antigen structure has further been reported to be present as core structures in oligosaccharides that have other sugar residues substituted for sialic acid[25].

Precursor T antigen has been described to be present on cell surfaces of carcinomata of diverse origin but not on healthy cells, and clearly interacts with the host's humoral and cell-mediated immune system[26,30,31]. Hence, the T antigen lends itself uniquely to the study of carcinoma cell surface antigens and the host response to it. T antigenic receptors are not accessible in healthy tissues to anti-T antibodies as they are shielded by O-glycosidically linked carbohydrates[20,23] but can be exposed upon desialylation by neuraminidase. The following scheme illustrates the relationship of the human blood group M to its precursors[26].



The Thomsen-Friedenreich antigen in cancer

The discovery of Thomsen-Friedenreich specific antigens in carcinomata of breast, colon and gastrointestinal tract but not in benign tumors or in healthy tissues has established a chemically reasonably well defined antigen apparently specifically associated with human carcinomata[22,26]. The appearance of precursor T specificity in a reactive form has been attributed to the possible incomplete or deranged biosynthesis or accelerated degradation of normal cell surface components[22]. The T antigen has always been found masked by sialic acid in normal healthy persons but [illegible] the cases of patients with breast, colon and gastrointestinal carcinomata where the [illegible] reactive T specific substance was found associated with malignancy[22,26]. Some of the mechanisms of antigen alterations in tumors were found to be due to:

1) the abnormalities associated with tumors in specific glycosyl transferases blocking the incorporation of sugar residues at or near the non-reducing terminus of oligosaccharide chains[32],

2) the inability of tumors to synthesize certain classes of glycoconjugates such as glycoproteins because of their inability to form the necessary precursor substances[33], and

3) the highly active enzymes found in tumors or tumor stroma such as glycosidases or protease acting along the tumor cell surfaces causing abnormal degradation of cell surface glycoconjugates[34].

Indirect support for the theories suggesting defective glycosyltransferase activty or excessive glycosidase activity has been provided by numerous reports of increased amounts of blood group antigen precursor substances in tumors[35].

A highly significant correlation has been described between the T antigen status and the histologic grade of tumors, where it was found that the majority of low grade tumors are cryptic T antigen positive while high grade tumors are generally T antigen positive or cryptic T antigen negative, thereby reflecting a parallelism between the morphological differentiation at

the light microscopic level and biochemical differentiation of the cell membrane[35]. The abnormalities of biochemical differentiation as reflected in the expression of the T antigen has been considered as a significant predictor of biological behaviour of some "look-alike" tumors which are histologically indistinguishable[35]. Relatively low grade tumors which are cryptic T antigen positive show a significantly lower incidence of tumor invasion and recurrence than low grade T antigen positive or non-cryptic T antigen tumors[35]. Furthermore, it has been postulated that the T specific antigens on cell surfaces of some cancers may be involved in the *in vivo* invasion and metastasis to healthy tissues since T-antigen determinants have been reported to be involved in the *in vitro* adhesion of invasive and metastic lymphoma cells to syngeneic hepatocytes[36].

Relationship between the T antigen and animal tumors

The Thomsen-Friedenreich antigen is not normally expressed on normal healthy mammalian tissues but can be found in a cryptic form masked by sialic acid residues in many common membrane integrated and soluble glycoproteins and glycolipids[37]. However, it has been reported that T antigenic determinants appear in an exposed form in a number of animal carcinomata and can be detected by both chemical and serological methods.

TA3 "spontaneous" mouse mammary adenocarcinoma

Intact TA3 cells and the ascites fluid from the TA3 tumor bearing mice of both the strain specific TA3/St and especially non-strain specific but much more invasive TA3/Ha sublines have been found to react with two agglutinins, *Vicia graminea* and anti-pneumococcus type XIV. Both of these agglutinins have serological specificity closely related to that of the anti-T agglutinin, peanut lectin[24,37]. It was further reported that both the TA3/Ha and TA3/St cells adsorb both human and animal anti-T antibodies[26], and TA3/St cells are killed by human anti-T in the presence of complement whereas TA3/Ha cells which possess much more sialic acid on their surface

were completely resistant. The neuraminidase removal of terminal sialic acid, which apparently interfered with complement fixation[40], from the surface of the TA3/Ha tumor cells resulted in the cells becoming fully susceptible to the cytolytic effects of human anti-T in the presence of complement[26]. Treatment of both the TA3 sublines, TA3/Ha and TA3/St, with neuraminidase followed by β-D-galactosidase rendered both sublines resistant to the cytolytic effects of human anti-T plus complement. Apparently, neuraminidase exposed the cryptic T antigen on the TA3/Ha and TA3/St cells and β-D-galactosidase removed the immunodominant carbohydrates that were reactive with anti-T from the cells[39]. The enzyme treatment with β-D-galactosidase was also seen to abolish the binding capability of the agglutinins *Vicia graminea* and *Arachis hypogaea* to the TA3/Ha cells[40]. A high molecular weight glycoprotein fraction called epiglycanin (glycoprotein 1)[41] was found to be present in high concentrations on the surface of the TA3/Ha tumor cells. It was determined that epiglycanin is composed largely of N-acetyl-galactosamine, galactose, sialic acid, serine and threonine which is similar in composition to the MN antigens[42]. Epiglycanin was further found to have blood group N-like specificity besides expressing the immunodeterminant structure of the T antigen on its surface[43].

RI lymphoma

The RI lymphoma tumor cell line which is derived from a radiation induced leukemia in a strain CBA mouse[44] has been reported to express the T antigen and have biological binding affinity for the anti-T agglutinin, Peanut lectin, *in vivo* and *in vitro*[45,46]. The RI lymphoma tumor model has been used as an experimental tumor model to study the *in vivo* biological distribution of T antigen binding radiopharmaceuticals[46]. Using radiolabeled Peanut lectin in this tumor model, scintiscans obtained at various time periods resulted in good tumor localization at 24 hours post-injection, and biodistribution studies reported high tumor to non-tumor tissue ratios at almost all time periods[45,46]. The localization index in this tumor model

with PNA, using F(ab'), nonspecific rabbit IgG as the nonspecific biodistribution control, was in the order of 11.5 at 24 hours and 8.4 at 48 hours post-injection[45]. Hence, this tumor model has been cited as a good biological model to study T antigen binding compounds.

ASPG-1 (rat) mammary adenocarcinoma

The Thomsen-Friedenreich antigen detected by its cross reactivity with peanut lectin has been found to be present on the ascites cell surfaces of the metastatic 13762 rat mammary adenocarcinoma but not on the solid tumor form from which the ascites form was derived[47]. Peanut lectin receptors have been reported to be present almost exclusively on the major sialoglycoprotein (ASPG-1) on various ascites sublines of this tumor[47,48]. This sialoglycoprotein, determined to have galactose, N-acetylgalactosamine and N-acetylglucosamine as the major sugars, has a composition different from that of epiglycanin on the surface of the TA3/Ha mouse mammary adenocarcinoma[41]. The T antigen bearing sialoglycoprotein, ASPG-1, has been indicated as the contributor to tumor cell survival in the ascites form and in the tumor cells' ability to metastasize and survive[47,48].

T antigen expression on human tumors

The Thomsen-Friedenreich antigen which is normally present in a cryptic form masked by sialic acid residues in many common membrane intergrated and soluble glycoproteins and glycolipids[37] has been claimed to occur in an exposed form in many human tumors, and thus has been commonly referred to as a tumor associated antigen[50]. A large variety of malignant, benign and healthy tissues as well as cell cultures examined by various immunoreactive methods for the presence of the T antigen resulted in the observation of T antigen expression in malignant tumors of the human breast[51], urinary bladder carcinomas[52], lung adenocarcinoma[53], colon and gastric carcinomata[54,55,57,58] and in some T cell lymphomas and leukemias[56]. The

exposed T antigen has not been, so far, found in sarcomas, malignant tumors of the central nervous system, benign tumors, thyroid carcinomas or tissues with non-cancerous disease[54-58].

Using an absorption assay to examine the presence of the T antigen by measuring the decrease in anti-T titre after incubating anti-T sera of known hemagglutination titre to neuraminidase treated red blood cells with various malignant and non-malignant isolated glandular and cellular membranes resulted in the finding that primary, metastatic and tissue cultures of ductal breast carcinoma were 87% (34/39), 100% (9/9) and 100% (4/4) T-antigen positive respectively compared to 9.5% (2/21) T-antigen positive with benign or healthy breast tissue samples[26,55]. T-antigen positive in this assay was designated when more than a 34% decrease in hemagglutination score in the anti-T titre was seen[55]. The T antigen specificity in a reactive form demonstrated in metastatic breast carcinoma but not in healthy and generally not in benign breast tissues[26,55] has been proposed to be due to the accelerated degradation or the decrease synthesis of terminal cell surface carbohydrate groups resulting in the exposure of the T antigen on breast carcinoma cells which are normally hidden or in a cryptic form in benign or normal breast epithelium[27].

Anti-T antibodies

Anti-T antibodies reported to occur in the sera of all humans and most animals[37,38,59] are apparently formed predominantly from the continuous antigenic stimulation by an individual's own intestinal flora[60] as it was found that numerous symbiotic gram negative bacteria isolated from the stools of healthy individuals presented the T specific antigen. The T specific antigen was also found in typhoid, *Corynebacterium parvum* and *Vibrio cholerae* vaccines[25,26]. It was reported that under the prolonged oral administration of antibiotics, the gastrointestinal flora diminished with the subsequent decrease in anti-T antibody levels in many individuals[26]. With the oral administration of *E. coli* O_{86} which possess T activity, an increase in

the levels of anti-T antibodies was observed. It was then further observed that *E. coli* O_{14} induced the *de novo* production of anti-T antibodies[34].

The majority of the anti-T activity in human sera was found to be associated with the euglobulin fraction that contains the IgM immunoglobulin (55%), while up to 30% was with the IgA immunoglobulin fraction. Weak activity was found in the fraction containing the IgG immunoglobulin fraction[38]. The immune system of most cancer patients has been reported to recognize the T specific structure on cancer cells as foreign[36], and these patients have been found to respond strongly, even if unsuccessfully as in the case of clinical cancer patients, against the T antigen on their cancer cells[55,57,59].

Humoral immunity

Anti-T antibodies predominantly stimulated by an individual's own intestinal flora[60] are mainly IgM immunoglobulins and can amount up to 14% of a person's total IgM[61]. Anti-T titre scores in human sera have been found to be severly depressed in a highly significant number of breast, lung and gastrointestinal tract carcinomata patients compared to normal and healthy individuals as well as to patients with fibrocystic disease, fibroadenoma or sclerosis of the breast[26]. It has been suggested that the depression of anti-T titre scores was due to the interaction of anti-T antibodies with T specific antigens or closely related glycoproteins and possibly glycolipids which are either attached to the cancer cell surface and/or released into the circulation[26]. Following surgery and removal of the primary carcinoma, there was a strong rebound or overshoot of anti-T antibody titre in the patient's sera[22,26,36]. Interestingly, a severe depresssion of anti-T IgM levels as compared with normal total IgM was observed in many cancer patients whereas there was no change observed in non cancer patients or healthy individuals. A strong rebound of anti-T IgM levels was seen in most of the patients following surgery of the primary carcinoma whereas anti-T IgG levels of these same patients were found closely similar to those of healthy people and non-cancer bearing patients before and after surgery[36,62]. These occurrences of the strongly elevated

IgM levels in patients following removal of their primary carcinoma implies dynamic carcinoma anti-T IgM interaction[56].

Cellular immunity

Cell mediated immune responses which are thought to play a role in the immune reactions against malignant tumors involve the direct role of lymphocytes and associated cells but are closely interdependent with the humoral system. Cell mediated immune responses towards the Thomsen Friedenreich antigen have been demonstrated in patients with various carcinoma *in vivo* and in some cases, *in vitro*[50,53,55] as it was observed that adenocarcinoma patients do recognize the carcinoma associated T antigen as being foreign[53]. A cell-mediated delayed immune response such as the delayed type hypersensitivity reaction to the T antigen (DTHR-T) involving cellular immunity similar to that of the classical tuberculin type reaction has been observed in patients with various types of carcinoma[53]. Breast carcinoma patients were seen to exhibit a positive delayed type hypersensitivity reaction to the T antigen following an intradermal injection of human erythrocyte derived T antigen in the arm contralateral to the breast lesion[53]. Positive DTHR-T was found in all patients with invasive breast carcinoma[26,64], in 88% of 101 patients with ductal carcinoma and in 43% of 30 patients with lobular or tubular carcinoma. More than 90% of the patients with histologically benign breast disease and all of the [illegible] persons tested responded with a negative reaction to the DTHR-T test [illegible] that the DTHR-T test was 78% accurate in terms of true positive in breast carcinoma patients tested, 100% accurate for healthy persons and 93% with 7% being false positives for patients with benign breast disease. Further studies resulted in the observation that the accuracy of the DTHR-T test varies with the type of breast carcinoma. Patients with ductal breast carcinoma had an 88% positive responses compared to a 43% positive responses in patients with lobular or tubular carcinoma[53]. The DTHR-T test was reported to be positive in all the 25 patients with lung adenocarcinoma and in 9/9 of the patients with adenocarcinoma of the body

cavities such as pancreas, esophagus, ovary, colon and salivary gland[53]. All seven patients diagnosed with small cell lung carcinoma and 3 out of the 5 patients with malignant melanoma also reacted positively to the DTHR-T test, whereas none of the 17 patients with malignant brain tumors, leukemia or Hodgkin's disease, sarcoma or thyroid carcinoma reacted[53]. All healthy persons and 48 out of 49 patients with other diseases including 23/24 with non-cancer lung disease reacted negatively to the DTHR-T test giving indication that the delayed type hypersensitivity reaction to the T antigen test is relatively accurate and a specific test for T antigen bearing cancers[53].

Using a leukocyte inhibition (LMI) test to determine cellular immunity to the T antigen *in vitro*, only 49% of the breast carcinoma patients gave positive readings compared to the positive reactions obtained in 16% of the patients with benign diseases and 1% of healthy persons tested, while not one of the 5 malignant melanoma and 1 glioma patients reacted positively[55]. Hence, this method of testing for the presence of cellular immunity to the T antigen is not as accurate and sensitive as compared to the DTHR-T test mentioned.

Diagnostic utility of anti-T sera

Human and animal anti-T sera have been utilized in various ways as diagnostic tools to detect T antigen bearing tissues both clinically and in laboratory conditions *in vivo* and *in vitro*. Using an absorption assay, in which the absorption of anti-T by the tissues expressing T antigenic specificity is measured by the decrease in anti-T score, a large variety of malignant, benign and healthy tissues as well as cell cultures have been tested for the presence of exposed T antigen. Various immunological methods utilizing human anti-T sera as the localizing agent have been performed to determine the presence of non-cryptic T antigenic structures on a variety of tumors such as breast carcinoma[31]. With these methods of detection, it has been reported that more than 90% of human adenocarcinoma tested was reactive with human anti-T sera[53].

The use of radiolabeled polyclonal anti-T antibodies in the radioimmunodetection of T antigen bearing tumors in animals has been investigated without much success. The results obtained in this study indicated a lack of specificity of the antibodies for the tumor cell antigens[45]. Some indication of tumor localization was observed with gamma camera imaging only at 72 hours post injection of the antibody in the T antigen tumor bearing mice. High background activity hampered any clear resolution and biodistribution studies performed reported prolonged blood background activity, low tumor to blood and low tumor to muscle ratios. It was concluded that the use of radiolabeled polyclonal anti-T antibodies was not fully suitable for clinical radioimmunoimaging of T antigen bearing cancers[45].

A plant lectin, peanut lectin (PNA), designated as an "anti-T agglutinin" since it gave similar immunological reactions as the anti-T antibody of mammalian sera[63] is gaining widespread use as a T antigen localizing agent. Several investigators have reported the preferential localization of peanut lectin for the T antigen in tissue and cells that exhibit T specific carbohydrates[30,63,94,95].

C. Lectins

Definition and classification

The term "lectin", taken from the latin word for "to choose, to select", was introduced by Boyd and Shapleigh[49] in 1954 when they thought that many of these carbohydrate-binding plant seed (glyco) proteins would be able to select human red blood cells according to their blood group. Unfortunately, only a limited number of lectins proved to be blood group specific and many carbohydrate-binding proteins have also been identified in a diverse range of organisms including bacteria, molds and algae, sponges, fishes, snails, eels, crabs and even in some mammals, besides plants[65]. Various proposals have been put forward by many investigators to define a lectin but up until now, the interpretation of the term has not been

uniform. Most investigators have agreed however that lectins are carbohydrate binding proteins (glycoproteins) which are able to agglutinate red blood cells and that they are of a non-immune origin. A definition for the term lectin which is presently adopted was that proposed by Goldstein et al[65] which defines a lectin as a sugar binding protein of non-immune origin that agglutinates cells and/or precipitates glycoconjugates.

There have been many attempts made to classify lectins immunologically. The use of the term "antibody-like substance" introduced by Boyd and Shapleigh[49] to classify lectins was deemed to be inappropriate[66] as the similarity of lectins to antibodies was found to be rather superficial. A purely descriptive classification term, "receptor specific proteins" was introduced to cover the wide range of reagents found in viruses, bacteria, fungi, plants, sponges, snails, fishes and other lower invertebrates[66] and furthermore, this term was selected over the more limited "sugar-specific" term proposed by Sharon and Lis[67] since it had already been demonstrated that a small number of the reagents were not inhibited in heamagglutination tests by simple sugars or oligosaccharides[61]. At present, it is considered to be rather difficult to classify a lectin explicitly in unambiguous terms with regards to specificity, unless as sugar binding proteins the lectins relegate to a section of a much larger group of receptor specific proteins which in turn could form part of the class of proteins referred to as affinitins[69]. The term "affinitin" was proposed as a collecting name for proteins (glycoproteins) such as antibodies, enzymes and lectins which contain combining sites and was based upon the community of certain chemical structures (combining sites)[69]. Since the introduction of the term "lectin" by Boyd[49], different names and classifications have been proposed by many investigators to label such reagents but as of yet, none of these names have been based on the still speculative *in vivo* functional role of these reagents, lectins[68].

Macromolecular properties

Many of the lectins used presently have been affinity purified to homogeneity on carbohydrate supported columns and many of these techniques of purification have been well

reviewed[72]. The purified lectins have revealed a diverse difference in physical and chemical characteristics[65]. There were no structural features that were common to the lectins studied except that the lectins were protein in nature[65]. Most lectins were found to consist of subunits which were identical or nearly identical in molecular weight[70], and the number of subunits per lectin molecule was found to vary. Most of the lectins were reported to be tetrameric in structure with a typical molecular weight of about 120 000 daltons (4 x 30 000)[70], although the molecular weight of lectins have been found to range from 36 000 to 335 000 daltons[71]. Some lectins were found to require the presence of metal ions such as Ca^{2+} or Mn^{2+} to exert a biological effect[65]. Many of the lectins are glycoprotein in nature but there are several notable lectin proteins such as the peanut lectin, concanavalin A and wheat germ lectin that lack any covalently linked sugars[72].

Physiological role of lectins in nature

Lectins derived from different sources have apparently been found to be involved in different physiological functions. Lectins, however, have been grouped together because they have in common the ability to agglutinate red blood cells but this reaction is a totally unphysiological reaction and hence, this grouping of lectins may be considered artificial[70]. It is at present difficult to assign a function to lectins in plants and animals as even the localization of the lectin within a plant is still not fully known[56,70]. Some investigators have assumed that lectins are associated with the protein bodies while others have found the lectins evenly distributed in the cytoplasm[70]. In most plants, the lectin consists of approximately 0.1% of the total seed weight ranging up to about 3% of the total seed's weight in some plants. Since lectins exist in such quantities in nature, it must be considered that lectins do have an important role in plant life[70].

Lectins in plants have been suggested to be involved in the transport, storage and immobilization of sugar, and may also function as protective agents by interacting specifically with the polysaccharide of plant pathogens[56,70]. Plant lectins have also been speculated to

facilitate the germination of seeds and promote early cell division and differentiation[56]. Other proposals put forward by investigators as to the function of plant lectins are that they may be involved in the extension growth of plants and they may act as enzymes in their natural enviroment[70].

Animal lectins are found to bind to galactose residues which usually constitute the subterminal sugar of serum glycoproteins[70]. Some of these animal lectins are also involved in the removal of glycoproteins from the circulation, while other animal lectins are thought to be involved in the organization of cells into tissues during the process of embryogenesis or may be involved in cellular communication and/or adhesion[56].

Lectin specificity

Lectins, considered to be a small group of protein agglutinins, possess the remarkable characteristics of having their agglutination activity inhibited by carbohydrates in a way that is similar to the glycosidases and other sugar binding proteins. The specificity of the lectin is considered rather relative as the lectin inhibitor referred to is often the "best" one or with the strongest inhibitory effect on the lectin-induced agglutination or precipitation reactions, hence implying that other inhibitors with different structures may be present although less efficient[73].

Stringent structural requirements usually exist for lectins to obtain optimal binding with their carbohydrate counterpart and it was often noted that the C-3 and C-4 hydroxyl groups of the sugars appeared to be involved in the lectin binding process[65]. D-galactose binding lectins have been generally found not to interact with any D-glucose or D-mannose binding lectins and vice-versa, hence demonstrating the critical involvement of the C-3 hydroxyl moeity in the determination of lectin specificity[65]. The saccharide binding sites of lectins were originally thought to accomodate only a single mono- or disaccharide, but some lectins have demonstrated extended saccharide binding sites which are composed of several subsites with each subsite being complementary for similar or different sugar moieties[82]. Both the peanut lectin (PNA) and concanavalin A are such examples of lectins having extended binding sites,

with PNA having a complimentary binding site for the heterosaccharide βDGal(1→3)DGal-NAc and the activity of concanavalin A can be inhibited by a series of mannose mono-, di- and trisaccharides comprised of α1,2-mannopyranosyl units that can be increased with chain length up to mannotriose[42].

The chemistry of lectin binding to complex oligosaccharides, glycoproteins and cells is considerably more complex than that of simple sugars as there are multivalent and secondary non-specific interactions involved which have been reported to be superimposed on the primary carbohydrate specific binding[43] and hence, the association constants that are involved with the binding of lectins and the disaccharide are of a much higher order of magnitude than that found in the binding between monosaccharides and lectins[71]. It may be further noted that there are reports of involvement of interactions other than the carbohydrate mediated ones which may participate in the recognition of glycoproteins by lectins such as the increasingly evident phenomena of hydrophobic bonding[73]. Many lectins with similar specificity for monosaccharides have been demonstrated to have large variability in the binding specificities to complex heterosaccharides[71]. Furthermore, some lectins have been shown to possess similar oligosaccharide residue specificities in addition to having their own binding ability to different sugars, thus making these lectins useful for investigating fine differences in carbohydrate structure of glycoconjugates having the oligosaccharide residue that these lectins are specific for as the terminal non-reducing ends[74]. The carbohydrate binding specificity of many lectins and their origin has been well reviewed by Zabel[76].

Relationship between lectins and antibodies

Similarities have been drawn between lectins and antibodies in many respects[71] such as the reversible specific interaction of the lectins with the carbohydrate bearing structure that induces agglutination or precipitation[43] which in some ways resembles the very specific and reversible antibody-antigen reactions. Like antibodies, lectins possess a combining site and, in most of the cases, the binding can be specifically inhibited by low molecular weight "haptens"

which could prevent the binding interaction. Most lectin interactions have been found to be inhibitable by simple monosaccharides but there are reports which have shown that the smallest molecular weight compound that can inhibit an antigen-antibody precipitation reaction is a dissacharide[77]. Major differences do exist between antibodies and lectins with respect to their characteristic properties[77]. Antibodies are heterogeneous in property in that more than one class of immunoglobulins are produced upon antigenic stimulation. Antibodies are essential with known physiological functions to the immune system. Furthermore, antibodies possess the unique property of having immunological memory antigenic stimulation. Lectins contrast the antibodies in that they are homogeneous and present without any known antigenic stimulation. The exact physiological functions of the lectins are still not known[77]. Lectins are present as cellular constituents and are not elicited by an immune response as are antibodies. The specificity range of lectins is usually confined to simple and complex carbohydrates[77] although protein and lipid receptors may exist whereas antibodies can be produced specifically to react against a variety of organic compounds such as amino acids, proteins and nucleic acids[71]. The antibodies produced usually have similar structural features whereas structural diversity is seen in all the purified lectins isolated from various sources[71]. Despite the differences between lectins and antibodies, lectins have been replacing antibodies in some clinical procedures such as in blood group typing[65,77] and in the mitogenic stimulation of lymphocyte transformation[77].

Uses of lectins

Lectins are found to be useful biological tools in various situations both *in vitro* and *in vivo*. Many cellular phenomena can be studied with the use of lectins since these lectins will interact specifically with the glycoproteins or glycolipids present on the surface of the cells that contain the carbohydrate chains which the lectins are specific for. The high affinity that lectins have for sugar residues and the possibility of labeling lectins with fluorochrome, ferritin, peroxidase, biotin or radionuclides make lectins a valuable tool in histopathological studies[15]. Thus, lectins have been used by many investigators to not only identify and localize glucidic

components of cells but also to follow any modification in pathological conditions. Reports of work performed with labeled lectins on various tissue sections under different conditions indicated that lectins present themselves as meaningful tools in diagnostic pathology[45].

Boyd and Shapleigh[49] proposed the original definition of lectins when they thought that many of the lectins would be able to select human red blood cells according to their blood group. Although it has been found that not all lectins are blood group specific, many lectins with well established blood group specificity have been found to be useful in the clinical typing and structural studies of blood group substances[78].

Lectins have the important properties of i) agglutination and precipitation, ii) characteristic hemagglutination patterns, iii) inhibition of hemagglutination, iv) agglutination of leucocytes and tissue cells and v) non-identity with enzymes[71] that make them interesting and characteristic tools to work with. The resultant agglutination and precipitation of cells that the lectins react with are easily detectable as a positive interaction or binding to the cell surface residues but there have been cases of positive cell interactions with lectins without any resultant agglutination or precipitation[71]. Studies have shown that the agglutination phenomena that results from lectin interactions is complex and involves many factors[71]. Agglutination was found to occur when the lectin involved is able to form multiple cross-bridging between opposing cells that it interacts with[71]. With the use of fluorescent microscopy, lectin receptor sites were found aggregated into clusters, not randomly distributed on cells following lectin interaction hence facilitating agglutination by allowing the lectin to form multiple cross-bridging between the cells[11]. There are studies that have reported that the cell agglutinability for lectins is markedly and significantly different between malignant and normal cells, embryonal cells and adult cells, mitotic and quiescent cells, and between normal and enzyme transformed cells[71,11]. The number of binding sites on normal and transformed cells has been reported to be similar by many investigators and the increased agglutinability of transformed cells has been postulated by various authors to be rearrangement or variations in the distribution of exposed sites[71,11]. Lectins are being exploited as a means of localizing

carbohydrate residues cytochemically at both the light and electron microscope levels and many lectins have also been investigated as potential histochemical reagents for localizing macromolecules with certain sugars on the basis of their specific binding affinity for these sacccharide residues[79-81]. Plant lectins have been used successfully for the detection, cell isolation and characterization of cell surface carbohydrates not found on normal cells but intensively expressed on carcinoma cells[10].

Purified and homogenous lectins have been utilized as columns in affinity chromatography for the purification of soluble and membrane glycoproteins and in the separation of glycoproteins and cells[82-84]. Many lectins and their immobilized derivatives, many of which are commercially available, are being used extensively as an affinity purification process and for the isolation of cell surface components from both transformed and untransformed cells in sufficient concentrations for chemical analysis[82].

With such *in vitro* demonstrated specific interaction of some lectins toward cell surface carbohydrates primarily associated with malignancy, lectins have been investigated as potential *in vivo* localizing agents. The lectin concanavalin A has been used as a potential *in vivo* tumor localizing agent where it was reported that concanavalin A was seen to selectively localize in certain tumors. It was further shown that concanavalin A possessed antitumor activity when it was shown to selectively agglutinate and kill certain tumor cells *in vitro*. Studies with concanavalin A carrying a chemotherapeutic agent in tumor bearing mice revealed that the concanavalin A-chemotherapeutic agent complex was far more effective in prolonging survival time than the use of either concanavalin A or the chemotherapeutic agent alone[83,84].

Another documented *in vivo* study of lectins is the use of radiolabeled peanut lectin to detect Thomsen-Friedenreich antigen bearing tumors in mice[45,46]. An impressingly high localization index was obtained in the T antigen tumors with the peanut lectin when compared with a $F(ab')_2$ fragment of a non-specific IgG molecule. High tumor to blood and tumor to muscle ratios were also obtained in the biodistribution studies for the T antigen tumor bearing mice but not in control mice bearing a non T antigen tumor. Scintigraphic images obtained at

various time intervals showed excellent images without the need of background subtraction in the animals[45,46]. Lectins have thus demonstrated some of their potential usefullness in *in vivo* situations. Lectins for *in vivo* applications should ideally be non-toxic to normal cells, non-mitogenic and should interact specifically with the carbohydrate residues that are primarily associated with the cell type in study such as malignant cells[45].

The peanut lectin agglutinin (PNA) has been reported in the use for the detection of T antigen bearing tumors since it has been found to give similar immunological reactions as the anti-T antibody of mammalian sera[63]. Several investigators have reported the preferential affinity of PNA for the immunodominant carbohydrate structure of the T-antigen[30,63,94,95]. Hence, the potential exists for radiolabeled peanut lectin to be used for the radioimmunodetection of T-antigen expressing tumors by gamma scintigraphy.

D. Peanut (*Arachis hypogaea*) lectin

Peanut lectin (PNA) has been designated as an "anti-T agglutinin"[63] because it was found that to give the same immunological response as the anti-T antibody of mammalian sera[94,95] which is responsible for T polyagglutination occurring in several bacterial and viral infections. The peanut lectin is found to be most specific for the carbohydrate structure βDGal(1→3)DGalNAc which happens to also be the immunodeterminant group of the Thomsen-Friedenreich antigen[27] that is normally found present in a cyptic form masked by N-acetylneuraminic (sialic) acid residues on a number of membranes and soluble glycoproteins in healthy individuals. Hence, the T-antigen occurring in a cryptic form on normal tissues will become available for PNA binding only after pretreatment of these tissues by neuraminidase. The T antigen became clinically significant when it was discovered that it was present in an exposed or non-cryptic form on human carcinomata but not on corresponding benign or healthy tissues[54]. This tumor associated antigen has since been reported to be present in an unmasked and reactive form in various adenocarcinomas such as lung, gastrointestinal tract and breast[53]. The peanut lectin which is a readily available protein that binds preferentially to the T

antigen has thus gained considerable interest as a potential tumor localizing agent and a possible carrier for chemotherapeutic or radioactive nuclides to T-antigen expressing tumors. *In vitro* immunochemical studies with PNA on various human tissue sections have shown marked differential binding patterns of the lectin in malignant, benign and normal tissue samples[94]. These *in vitro* studies strongly support the use of radiolabeled peanut lectin for the *in vivo* localization in T antigen bearing tumors.

Macromolecular properties

The molecular weight of affinity purified and homogeneous peanut lectin was measured by various methods such as sedimentation velocity, gel filtration and sedimentation equilibrium centrifugation and was estimated to be around 110 000[87,90] although some investigators have reported a lower value of 98 000 ± 3 000[96] for the intact molecule. The discrepancy found in the reported values has been attributed to various possible factors in the methods and materials used by the different investigators such as methods of extracting the peanut lectin, concentrations of the lectin used[96] and also the possibility of isolectin variation[97], all of which may contribute to the variances in the molecular weight determination of the peanut lectin. Peanut lectin was found to be a relatively acidic molecule with a pI of 5.95[90], a hydrophilic and a compactly folded globular protein[96] composed of four identical monovalently linked subunits each with molecular weight of 27 500[87,91]. This tetrameric structure was found to be pH dependent as it was observed that with the gradual decrease of pH to about 3.0, the intact molecule reversibly dissociates from a tetramer to a still globular 48 000 dalton dimeric species which lacks sugar binding capacity[96]. Furthermore, the intrinsic sedimentation coefficient for the intact PNA tetrameric structure at pH 7.4 was found to be at 5.7 ± 0.1s and decreasing reversibly to a value of 3.8 ± 0.2s upon dissociation to the dimeric structure at pH 3.0[87,96]. Under denaturing conditions or in the presence of detergents, the PNA molecule was found to dissociate into 4 single identical 27 500 dalton components with identical sequencing of the last five NH_2-terminal amino acids[87,90,96].

X-ray diffraction pattern studies with crystallized PNA revealed the lectin molecule to be an asymmetric orthorhombic crystal with about 57% of each PNA molecule occupied by solvent and the hydrodynamic radius of the molecule was estimated to be around 35.5 ± 1.5 Angstroms[95]. Equilibrium dialysis performed on PNA revealed the presence of four binding sites per intact molecule. A much larger enthalpy change was involved when the PNA reacted with a disaccharide than with a monosaccaride[99] and the entropy contribution was reported to favor considerably more towards the monosaccharide than the disaccharide. The thermodynamic parameters involved in the binding process of the PNA molecule to the structure βDGal(1→3)-D-GalNAc has been determined as being -78 ± 5 kJ mole^{-1} for the enthalphy ΔH° and -177 ± 16 Jmole^{-1} K^{-1} for the entropy S°[99]. Ultraviolet spectral analysis of the peanut lectin revealed a small peak occurring at 290nm and a double maxima at 277nm and 283nm[96]. In the presence of saccharides which PNA is specific for, a near UV circular dichroic spectrum was observed that was due to a saccharide induced transition[96]. The extinction coefficient for PNA at 280nm was determined to be 0.96[96] while the absorption coefficient $A_{1\%}$ was found[97] to be 7.7 cm^{-1}.

The peanut lectin, unlike most other lectins which are glycoproteins, does not contain any covalently bound sugars. The amino acid composition revealed the PNA molecule to be rich in acidic and hydroxyamino acids, low in methionine, histidine and tryptophan, and devoid of cysteine and cystine[97] although other investigators have reported different results in their amino acid composition determination of PNA[90]. Metal analysis of PNA by atomic absorption analysis indicated that each subunit of the PNA molecule contains one Ca^{2+} and Mg^{2+}/Zn^{2+} atom (0.78 mole Mg^{2+} per subunit and 0.11 mole Zn^{2+} per subunit)[100].

Isolation and purification of the Peanut lectin

Most of the protein components in peanuts can be extracted with a 10% (w/v) sodium chloride solution, and these extracts are usually found to consist of two major protein components, arachin and conarachin[46]. The anti-T activity was originally seen in such crude

saline extracts[63]. With further extraction of the protein containing saline solution using an organic solvent such as petroleum ether, most lipid components that may have been carried over in the saline extract are removed[87,90]. The petroleum ether extract is then usually dried, resuspended in normal saline, washed and the supernatant retained[87]. The proteins found in the supernatant solution can be salted out using a salt such as ammonium sulfate. The resulting insoluble fraction collected is usually centrifuged before being redissolved for dialysis or ultracentrifugation[87,90]. The clear supernatant solution will contain the peanut lectin protein besides a number of other peanut proteins[87] and the peanut lectin can then be separated from the other proteins in solution by affinity chromatography which exploits the capability of the lectin's sugar binding specificty[72]. At present, there are various affinty absorbents available to purify PNA. These affinity absorbents are usually a carbohydrate ligand which PNA is specific for that are insolubilzed in some manner such that upon the application of the purified protein solution, it will reversibly bind PNA and the bound PNA can then be eluted off the column by applying a solution of sugar that will compete for the same binding site as PNA and hence will displace PNA from its bound position[72]. Other affinity purification methods exist that do not utilize a carbohydrate ligand to purify PNA[95,101] and one such example is the use of Rivanol® (2-ethoxy-6,9,-diamine acridine lactate) which will precipitate all the proteins except immunoglobulin G in the serum. The use of Rivanol® on crude saline extracts of peanuts have resulted in the selective precipitation of most of the peanut proteins while leaving the anti-T protein in solution[91]. This is a rapid and simple method to perform and the PNA thus prepared was found to be serologically as active as the PNA that was purified by some of the affinity chromatographic processes[91].

Isolectins : Multiple molecular forms of PNA

Affinity purified peanut lectins that were considered to be homogeneous by most protein purification/separation techniques were reported to consist of several isolectins with very similar isoelectric points as demonstrated by polyacrylamide gel electrophoresis and

isoeleetric point focusing[97,102]. All of the isolectins obtained were reported to consist of subunits of similar molecular weight (28 000). These isolectins agglutinated neuramindase-treated erythrocytes equally well and were immunologically cross-reactive to antibodies raised from any genotypic strains of *Arachis hypogaea*[97,102]. The protein peanut lectin was reported to be present in 4556 genotypes of the peanut, *Arachis hypogaea*, and in 65 genotypes of species related to *Arachis*[97]. All but four of the wild *Arachis* species tested had seeds containing PNA and these four lines of seeds that lack the presence of PNA were reproducibly found not to form any immunoprecipitates with anti-PNA IgG antibody or agglutinate desialyzed human erythrocytes. Further tests showed that these four lines were devoid of any hemagglutinating lectins as well[97]. Peanut lectin preparations from the peanut variety Shulamit were observed to have mitogenic properties toward desialyzed lymphocytes[103] but this mitogenic activity has not been found in other unspecified genotypes of *Arachis hypogaea* and the difference observed was reported to be apparently due to the heterogeneity of PNA isolectins, a phenomenon which has been found to be dependent upon the variety of the peanut used as the source of seed meal[97].

Stability of the Peanut lectin

Proteins have been known to denature and/or lose their original biological properties under changes of pH and temperatures. The protein properties of PNA have been found to remain relatively resilient as certain changes in pH and temperature conditions were reported not to bear any effect on PNA[101]. Using ultraviolet spectral analysis, the structure and conformation of the peanut lectin protein was found to remain constant in the concentration range of 0.5 - 2.0 mg/ml, in the temperature range of 0.8 - 52.0° C and in the pH ranges of 3.0 - 10.75[101]. Cryoinsolubility, however, has been observed for the peanut lectin and has been reported to most likely occcur at higher concentrations of the protein, at temperatures lower than 4° C and in the pH range of 5 - 9. This cryoinsolubility of PNA has been attributed to most likely be the individual solubility properties of the lectin itself as neither conformational changes of PNA at low temperatures nor polymerization of the PNA protein was observed[101].

Cryoinsolubility of PNA was found to be partially reversible and this phenomena can be totally inhibited by galactosides that are specific ligands of PNA with the disaccharide βDGal(1→3)αDGalNAc being the best inhibitor of this low temperature precipitation[101]. Residues localized in the region of the sugar binding site or within close vicinty to it are considered to be responsible for the aggregation and these residues in the binding of saccharides become masked or locally reorganized, hence preventing aggregation[101].

A shelf life of over 6 months without any substantial loss of hemagglutination activity has been reported for crude saline extracts of peanuts stored at 20° C[31] and affinity purified PNA that has been reconstituted after lyophilization to a concentration of 1 mg/ml in the presence of bovine serum albumin was found to be stable for 2 years stored at 4° C[31]. Repeated freezing and thawing of isolectin PNA solutions were found not to have any effect on the biological activity on the isolectin composition of the peanut lectin[97]. Further stability studies performed on PNA showed that the shelf-life of PNA at -20° C was at least six months with no resultant loss in agglutination activity of the protein[31].

Carbohydrate binding specificity of the Peanut lectin

Crude saline extracts of peanut (*Arachis hypogaea*) are known to agglutinate human red blood cells that have been previously exposed to neuraminidase[30,63] and these desialyzed erythrocytes of the human ABO blood group types were found equally well agglutinated[87]. This agglutinating activity of the peanut extracts could be inhibited by sugars such as lactose and galactose[30,63], and amongst the monosaccharides that have been tested for the inhibition of heamagglutination by PNA, D-galactose and α- and β-D-galactosides were active while high inhibitory activity was found with the disaccharide 2-acetoamido-2-deoxy-3-Oβ-D-galacto-pyranosyl-D-galactose (βDGal(1→3)GalNAc), and with desialylated glycoproteins such as α_1-acid glycoprotein, fetuin, glycophorin and the human blood group NN or MN antigen[87] all of which contain the core structure βDGal(1→3)DGalNAc upon desialylation. Besides finding the disaccharide structure which PNA is specific for in many soluble glycoproteins among

soluble membrane glycocompounds, the structure has also been found in glycosphingolipids GM1 and asialo GM1[104]. Direct binding was observed with the oligosaccharide structure of asialo GM1 but no direct interaction was found to occur with the GM1 structure[104]. There has been considerable overlap seen in peanut lectin receptor positive cells and asialo GM1 positive cells[105]. The disaccharide βDGal(1→3)DGalNAc found to be most specific for the peanut lectin has also been identified as the immunodominant carbohydrate structure of the Thomsen-Friedenreich antigen, and furthermore the peanut lectin was found to give the same immunological reaction as the anti-T antibody of mammalian sera, hence PNA has been designated as "anti-T agglutinin"[63].

The binding activity of affinity purified PNA was reported not to be inhibited by terminal N-acetyl-D-galactosamine in contrast to most of the D-galactose specific lectins[17,91]. Also, the peanut lectin is one of the few lectins that are able to accomodate more than a single glycosyl residue at its combining sites[65]. An extended binding site has been proposed for PNA when it was observed that PNA interacted specifically with the disaccharide βDGal(1→3)GalNAc with an affinity that was fifty times greater than the galactosyl monosaccharide[17,91]. The structural requirements of saccharides for the interaction with the carbohydrate binding site of PNA have been made on the assumption that the heamagglutination inhibitory activity of the saccharides reflect the affinity of the peanut lectin for the saccharides[17,106]. The following are the conclusions that have been drawn with respect to the importance of the configuration of atoms necessary for the binding interaction of the peanut lectin with the receptor site on the saccharide.

1. On C-6, an extracyclic chain was found necessary for the carbohydrate lectin interaction and the orientation of the hydroxyl group on C-6 is essential. A hydroxymethyl group on C-6 is necessary for binding as any substitution was found to decrease the strength of the association[17,106,107].

2. A free hydroxyl or a free amino group at C-2 is required for interaction and although a C-2 hydroxyl at an equatorial position is not essential for binding, the

axial position of this group was reported to decrease the binding[87,106].

3. A free axial hydroxyl on C-4 has been found necessary for binding[106] and the configuration of the C-4 terminal residue should be similar to D-galactose for activity[87].

4. A methylated O(1) on the C-1 favors association, and among the methyl-D-galactosides differing only in the configuration about the anomeric C-1, only a slight preference was observed for the α anomer. The O(1) was reported to be involved in glycosidic bonding and the second residue in the oligosaccharides studied appeared to be involved in the association. The β(1-4) linkage appeared to give better binding than the α(1-6) linkage, hence suggesting that the configuration on the C-1 is important for binding[87,106].

Sugars in the pyranose form were found more effective in blocking the agglutination activity of PNA than the other forms[87]. The sequence C(4)-O(4), C(5), C(6)-O(6) of the galactopyranosyl ring is postulated to be involved with the interactions in the carbohydrate binding site of PNA[87]. The actual lectin-glycoprotein interaction is far more complicated than that elucidated by precipitin-inhibition assays with various carbohydrates as there are other factors involved in the interaction that are not only inside the combining site itself but also at other parts of the molecule[101]. Peanut lectin, like most other plant lectins, exhibits a near UV circular dichroic spectrum that undergoes a saccharide induced transition in the presence of the saccharides that PNA is specific for[96]. The two positive peaks of PNA observed on the ultraviolet spectral analysis at 285nm and 279nm are in agreement with the assumed involvement of the tyrosine residues in the lectin-sugar interactions[107]. These peaks in the UV absorption change upon interaction with the sugars. Quantitative differences such as association constants and thermodynamics are found to exist in the binding between the peanut lectin and different sugars which can be determined by the method of UV difference spectroscopy[107].

Peanut lectin versus anti-T antibody

Based on the similarity of the immunological reactivity of PNA and anti-T antibodies, the peanut lectin has been termed as an "anti-T agglutinin"[63]. It soon became evident that the immunological activity of the peanut lectin towards the Thomsen-Friedenreich antigen is due to the Thomsen-Friedenreich immunodominant disaccharide βD-Gal(1→3)GalNAc[27]. Peanut lectin which is being widely used as a cell surface probe[111] and in routine clinical use in blood banking for the detection of the T antigen[152] has some semblence to human anti-T antibody in its reaction with desialylated erythrocytes[63] where βDGal(1→3)GalNAc is the prominent terminal group[23,113]. Although similarities have been drawn between the specificity of PNA and "naturally" occurring human anti-T antibody, reports have shown that differences do exist in the binding pattern of PNA and anti-T antibodies[109] both in animal and human tissues indicates that the specificity of these two, PNA and anti-T antibodies, are not identical. Several distinctions have been made between PNA and anti-T antibodies[27] and these differentiations have to be taken into consideration when interpreting histochemical and serological results[27,91]. The list below outlines the possible receptors that have sites for the peanut lectin whereas anti-T sera has affinity for only the "true" Thomsen-Friedenreich receptors (number 1 of the list):

1. The "true" Thomsen-Friedenreich receptors such as those found present on the glycophorin of red blood cells which are detectable by both anti-T sera and peanut lectin.

2. Pseudo Thomsen-Friedenreich receptors which are detectable only with the peanut lectin because they have only the T disaccharide such as the asialo ganglioside of the GM1 type and other glycoproteins with such alkali-labile chains[27].

3. Cross-reacting receptors which under certain circumstances the peanut lectin has been seen to react with such as β-galactose(1-3) galactosido structures found in *Helix pomatia* galactan and *Achatina fulia* galactoprotein, and N-actyl-lactosamine

structures found in α_1-acid glycoprotein, edible bird's nest glycoprotein and normal rabbit and guinea pig erythrocytes[37].

The immunochemical activity of PNA measures, in addition to the immunoreactive T, structures that are not involved in the T- anti-T immune response of carcinoma patients against their cancer and indeed, some of the PNA reactive structures are unrelated to cancer[34,51,109]. The peanut lectin requisite for complementary structures differ rather profoundly from those of the human anti-T antibodies[23,87,91,110] where the latter has an absolute requirement for terminal βDGal residues preferably linked 1→3 to GalNAc. Although PNA has high affinity for the structure, it also reacts with galactose and with methyl α-Gal better than the β anomer[87,91]. In further contrast to human anti-T, PNA reacts with ordinary D-galactopyranose and with both the α- and β-linked D-galactopyranose residues. The peanut lectin has also been reported to react with D-galactosamine, α-linked D-glucosamine and even D-fucose[55]. There are reports of slight but definite inhibition of PNA activity by substances that are lacking the PNA receptor disaccharide βDGal(1→3)GalNAc such as the Pneumococcus type XIV polysaccharide and the Helix pomatia galactan[114]. PNA was further seen to be strongly precipitated by α-1 acid glycoprotein (orosomucoid) from serum, a mucus glycoprotein from edible swallow's nest and a glycoconjugate from the albumin gland of the snail Achatina fulica all of which have been found negative for the PNA receptor disaccharide[114]. Red blood cells obtained from guinea pig, rabbit and rat (pronase treated) were agglutinated by PNA without any treatment by neuraminidase and the glycoprotein fraction isolated by phenol-saline extraction of rabbit erythrocytes was found serologically and by means of GLC to have "free" PNA receptors only after neuraminidase treatment[114]. Hence, the peanut lectin has some superficial resemblance to human anti-T in its actions[63] but the requisites for complementary structures of PNA differs from the human anti-T antibody population[55]. The lectin PNA does appear to be quite unspecific in its actions which is in sharp distinction to human anti-T antibodies[23,87,91,115].

The peanut lectin has been shown to be substantially more tolerant to neuraminic acid during extensive desialylation in the immediate vicinity of the T antigen's immunodominant repeating structures, βDGal(1→3)GalNAc[53,61]. With gradual desialylation of human erythrocytes, a significant inhibition in the agglutination of T-activated erythrocytes by PNA was observed with the human erythrocyte M and N antigen whose neuraminic acid content has been reduced by 20 - 25%[53,61]. Human erythrocytes whose M and N antigens have only 5 - 8% of their total neuraminic acid content, were found to be as active in inhibiting PNA agglutination as those from which all removable neuraminic acid has been cleaved[53,61]. Inhibition by human anti-T, however, was not achieved when these erythrocytes had more than 12% total neuraminic acid present[53]. During a stage of desialylation of erythrocyte M and N antigens, maximum inhibitory activity toward PNA was reached whereas only 10 - 20% of maximal activity toward human anti-T was obtained with the partially desialylated erythrocytes[53]. Faint activity was first recognizable with human anti-T when 50 - 55% of the neuraminic acid was released from NN and 65 - 70% was released form MM antigens[61]. At this level of desialylation, the NN and MM antigen had about 50% of the maximal activity toward PNA[61]. Using the glycoprotein fraction 1, epiglycanin of the TA3/Ha murine mammary adenocarcinoma which has 13% neuraminic acid as a comparison, a thousand fold difference in inhibitory activity was found toward PNA rather than human anti-T[61,53]. Epiglycanin was also 5 times as active with PNA compared to the authentic T antigen with less than 2% neuraminic acid[50] but the native epiglycanin was found to have less than 0.5% of the activity of the T antigen with human anti-T[53]. Carcinomatous and to a lesser degrec benign and healthy breast glands have been found to adsorb the peanut lectin whereas human anti-T was only seen to adsorb onto carcinomata provided no autolysis had previously occurred[54,55].

It seems that the specific receptor for PNA is a β-D-Gal residue which is revealed *de novo* as a result of neuraminidase treatment of erythrocytes and that the antigen revealed by this treatment is a Thomsen-Friedenreich antigen which also possesses a terminal β-D-Gal residue but involving as well the protein vicinal groups which distinguishes it from the β-D-Gal

residues present as T antigens in glycolipid moiety of the erythrocyte surface and is independent of β-D-GalNAc[30]. Peanut lectin which precipitates macromolecules that carry complementary haptens in contrast to human anti-T antibodies[50,53,98] has been substituted in many important immunohistochemical studies for the human anti-T antibodies because of its readily recognizable and avid reactivity. Some investigators have suggested that any structure that reacts with the peanut lectin may be designated the Thomsen-Friedenreich antigen[51,61,94].

Biological binding affinity of the Peanut lectin

Since the initial discovery of the heamagglutination properties of PNA on desialylated erythrocytes and its reported similar immunological response to the anti-T antibody of mammalian sera[30,63,94,95], PNA has gained widespread use as cell surface probes[111] and various investigators have reported the numerous immunological uses of the lectin on a variety of biological conditions especially on the localization of T antigen bearing tumors.

Red blood cells (erythrocytes)

Peanut lectin is in routine use now in many blood banks for the detection of the T antigenic disaccharide[71] βDGal(1→3)DGalNAcα1-linked which is the mucin type carbohydrate sequence normally found in a cryptic form on human erythrocyte membranes[21] and has been identified as the core structure of the human blood group MN determinant on red blood cells[119]. The distribution of PNA positive cells in human peripheral blood was found to be about 5.0% of which most appears to be monocytes but this distribution has been found to be lower in the murine system[116]. Purified PNA has been reported to agglutinate neuraminidase treated red blood cells irrespective of their ABO blood group. The Thomsen-Friedenreich antigen can be demonstrated both serologically and chemically to be a cryptantigen on the membrane of erythrocytes[21]. It has been determined that human erythrocyte membrane glycoprotein contains about 20.8μmol of N-acetyl galactosamine and about 48.7μmol of sialic acid per milligram of erythrocyte glycoprotein[117]. Strong inhibition of PNA agglutination of desialyzed

erythrocyte were observed with human anti-T sera while no activity was seen at all with the native form of the erythrocyte glycoprotein[117]. Upon the reductive cleavage of the glycoprotein from human erythrocyte membranes, a tetrasaccharide structure, N-acetyl neuraminyl-(2→3)β-D-galactosyl-(1→3)-[N-acetylneuraminyl(2→6)]N-acetyl-D-galactosaminitol was released[21]. This structure is the major alkaline-labile oligosaccharide found on human erythrocyte membrane[118] and is also the required structure for the activity of the blood group M and N[119]. The immunodominant T antigenic structure, βD-Galactosyl(1→3)-N-acetylDgalactosamine was shown with subsequent studies[120] to be the alkali-labile disaccharide asialo form of the tetrasaccharide isolated by Winzler[118] from the human erythrocyte membrane. Further studies with the glycoprotein of erythrocyte membrane isolated from animals such as the pig and the cow showed that the C-6 position of the N-acetyl galactosamine in the alkali labile oligosaccharide is substituted by sialic acid[28,118].

Some investigators have suggested that there are two types of T antigens exposed on red blood cells upon the removal of terminal sialic acid; those with terminal D-galactose and those with terminal N-acetyl-D-galactosamine of which only the former is recognizable by PNA[30,87]. Human senescent red blood cells that have about 70% the sialic acid content of young cells or those cells that have an extruded nuclei which contain less than 50% of the anionic groups of newly formed reticulocytes are not agglutinated or bound by PNA after the removal of surface sialic acid[21]. The cause of this phenomena in aged red blood cells in circulation is probably due to the *in vivo* cleavage by glycosidases such as β-galactosidase of exposed D-galactose residues for which PNA and anti-T sera are specific for[21]. Aging of red blood cells *in vivo*, however, does not unmask N-acetyl-D-galactosamine residues[21]. Asialo glycopeptides derived from human MM erythrocytes were found to have as much as 10 times the activity of NN derived ones in terms of T activity. This difference in activity was attributed largely to the presence of O-glycosidically linked carbohydrate substitution on the NH_2-

terminal amino acids on MM glycopeptides in which NN glycopeptides are lacking[121].

Lymphocytes

Lymphocytes are a type of white blood cells that are normally found in the peripheral circulation and are formed in the lymphoid tissue. Small lymphocytes are thymic derived and are found in the circulation and in germinal centres of lymphoid tissue while large lymphocytes are gut-associated lymphoid stem cells which can further differentiate into the immunoglobulin producing plasmacyte. The basis for the development and maintenance of humoral and cellular immune mechanisms result from the interaction of T and B lymphocytes.

Peripheral blood lymphocytes of humans, rats, mice and guinea pig have all been found not to bind PNA unless they have previously been exposed to neuraminidase[103,116]. PNA has also been seen to stimulate mitogenic activity in neuraminidase treated human and rat lymphocytes[103]. However, in the lymphoid cell population, exposed PNA membrane receptors are seen to occur on immature thymocytes and some neoplastic lymphocytes[87,116]. In the murine system, PNA receptors were found in an exposed form on the cell surfaces of cortisone sensitive thymocytes and suppressor T cells[123] and this discovery has been exploited in the separation of suppressor T cells from the cell population[122]. Peanut lectin positive cells appear to be present during the very early stages of mouse ontogenesis of the thymus and spleen, and some of these PNA positive fetal cells have been identified as T cells[116]. The potential thus exists for the use of PNA as a marker in the studies of T cells in their early development and differentiation[116]. Most of the thymus generated cytotoxic T cells were reported to be PNA receptor positive and further investigations revealed that the majority of the cytotoxic T cells that had been derived from PNA receptor negative thymocytes or lymph node cells were PNA receptor positive[124]. Hence, conclusions that had been drawn from these studies were that activated T cells acquired PNA receptors upon enlargement and these same receptors were lost upon reversal to

the small sized lymphocytes[124]. With these observations, PNA receptors can no longer be considered as unambiguous markers of T cell immaturity outside the normal thymus[124]. The peanut lectin thus provides a means of studying the human thymocyte subpopulations as well as T cell differentiation among the various organs[116]. The rosette fractionation by PNA is a method that is simple and useful in isolating human suppressor T cells[122]. Approximately 5% of the cells in human peripheral circulation are PNA positive; of these less than 1% are lymphocytes while monocytes have been found to be responsible for most of the population of PNA positive cells[116]. These figures obtained are in contrast with the results from mice studies where it was determined that about 24% of murine peripheral blood mononuclear cells have PNA receptors and most of them are T cells[116].

Analysis by means of gas chromatography revealed that platelet plasma membranes released the disaccharide β-D-galactosyl(1→3)-N-acetyl-D-galactosamine after the platelets had been treated with alkaline borohydride after desialylation[125]. Immunological detection with both the peanut lectin and anti-T serum further confirmed the presence of the disaccharide which is also the immunodominant carbohydrate structure of the Thomsen-Friedenreich antigen only on platelets that had been treated with neuraminidase[125].

Peanut lectin binding sites have been found on cell membranes in various lymphoblastoid lines that have either T- or B- cell characteristics[126]. A large variety of lymphoblastoid cell lines have been investigated for the presence of binding sites for PNA on their membranes[126]. Among the cell lines tested, it was noted that the cell lines from Burkitt's lymphoma on the average had more PNA binding sites on their cell membranes than the other lines studied followed by the T cell lines from leukemias. It was further seen that normal peripheral blood B lymphocytes that had been infected by the Epstein-Barr virus have the lowest number of binding sites and some of the cell lines in this category were found devoid of any available PNA binding sites[126]. The

results thus obtained with the studies on the various animal and human cell lines gives an indication of the potential and practical use of the peanut lectin in the detection, isolation and characterization of lymphoid cell subsets[126].

Binding affinity of the Peanut lectin to tumor cells

The peanut lectin based on its similar immunological reactivity to the anti-T antibodies in mammalian sera has been used in a number of studies to detect the presence of the T antigen in various tissue sections and also *in vitro* cell cultures using a variety of immunological methods.

Mammary carcinoma

The T antigen has been rather well documented to be present on breast carcinoma in a reactive form but not on corresponding healthy and benign breast tissues[50,54,58,59]. The T antigen has been found in large amounts in the cytoplasm and cell membranes in most breast carcinoma whereas the luminal membrane of the ducts and lobules with the occasional intraluminal secretions in benign breast tissues also showed some positive T antigen presence[35,109].

The difference in the binding pattern of PNA observed with benign and malignant cells has been postulated to be due to the dependence on cellular localization of the immune response to the T antigen or T antigen-like substance[109]. In tissue sections, various benign breast tumors revealed free as well as cryptic T antigens upon incubation with labeled PNA, with the apical region of the epithelial cell membranes giving the highest reactivity[129]. There were some reported observations of masked and unmasked T antigen in the cytoplasm in some benign tumors. Peanut lectin localization was seen most pronounced on the epithelial membrane of the intraductal papillomas while the fibrous stroma of both fibroadenoma and cystosarcoma phylloides were PNA receptor negative even after neuraminidase treatment[129]. A small amount of PNA reaction was seen in the cell membrane of invasive and *in situ* breast carcinoma and it

was further observed that the majority of PNA receptors in this condition were covered with neuraminic acid[26,129]. The presence of the Thomsen-Friedenreich antigen in carcinoma lobulare *in situ* which is regarded as being derived from myoepithelial cells has been considered associated with a secretory condition[129]. In normal breast parenchyma, positive reactions with labeled PNA have been noted along the luminal surface of the breast epithelium in the lobula as well as in the ducts. Furthermore, the secreta found lying within the ducts were also rich in free T antigen receptors without neuraminidase treatment and upon incubation of these tissue sections with neuraminidase more T antigen receptor sites were revealed[121,129]. The number of free receptors present were found to be dependent upon the secretory condition of the epithelium[129] and, chemical as well as serological analysis done on the milk fat globule membrane glycoproteins complemented the histological demonstration of the presence of free T antigenic receptors on the secretion of the lactating gland[117]. Similar findings have been reported in fibrous and fibrocystic diseases in comparison to normal breast parenchyma with regards to PNA receptors. Free T antigens were seen in the apical region of the epithelium while a great range of PNA positive reaction were noticed in cystically enlarged ducts. Although the secretion of some of the cysts were found to have few PNA receptors, enlarged ducts were seen to give strong positive reactions with PNA[129].

Most undifferentiated breast carcinoma have been reported to be completely negative for the T antigen both with and without neuraminidase treatment[35,94,109,131] but some investigators have observed some slight amount of binding of PNA to undifferentiated carcinomas of the breast[129]. Neuraminidase treatment of benign and malignant breast tissue have, however, been found to increase the intensity of PNA binding. Widespread membrane and cytoplasmic binding of PNA has also been seen with neuraminidase treated benign breast tissue[109]. With human mammary carcinomas of different histological grade such as adenocarcinoma, infiltrating lobular carcinoma,

infiltrating ductal carcinoma, carcinoma soidum and medullary carcinoma, an unsuccessful attempt was made to correlate the presence of PNA receptors to the histological grade of the tumors[131]. Using light microscopy and peroxidase labeled PNA, PNA reaction was observed not only with all the tumor cells but also in parts of the stroma in all of the various carcinomas mentioned above. Again, an unsuccessful attempt was made to correlate the degree of PNA reactivity in the stroma with the histological type of tumor[131]. Presently, the biological significance of the absence and presence of PNA receptors in such structures is still unknown[129] although some investigators have proposed that the T antigen that can be detected by PNA reactivity may be involved in the modulation of the host immune response to breast carcinoma and have considered that the T antigen located on the luminal membrane in benign breast tissues and in well differentiated areas of neoplastic tissue is "outside" the body or in an immunologically priveleged site[131]. Most of the PNA studies cited in the literature have been performed on tissue sections and/or cells. While many of these *in vitro* studies have turned up PNA receptor positive reactions, these same tissues may not bind PNA under *in vivo* conditions as these sites may not be accessible to PNA *in vivo*. The process of tissue sectioning may also reveal PNA receptor sites that are poorly vascularized or immunologically priveleged[51,109]. Furthermore, the processing of tissues with reagents such as formalin may cause autolysis of the tissue resulting in the possible exposure of normally cryptic T-antigenic structures[110].

In animal studies to determine the involvement of hormone receptors and PNA receptors, it was found that in the presence of oestrogen, progesterone, dihydrotesterone or cortical receptors in the concentration greater than 25fmol/mg cell protein, a 68% positive PNA reaction was noted in primary tumors and a 60% positive PNA reaction in metastic tumors[93]. In comparison to tumors with little or no hormone receptors (less than 25fmol/mg cell protein) only 23% of the primary tumors tested had PNA reactivity with no activity noted in metastatic tumors[93]. In the presence of

oestrogen receptors alone on primary breast tumors there was a 45% positive reaction observed and a 25% positive reaction with the metastatic breast tumors. With ovariectomy of the animals, an abolition of PNA binding site expression resulted, but upon the administration of 17β-oestradiol to these animals, large amounts of free as well as neuraminic acid covered PNA binding sites were restored[93,105], thus indicating that the ovarian hormones are important for the expression of PNA receptors in hormone dependent neoplastic mammary tissue[105]. Further studies implicated the involvement of oestrogen receptors in PNA receptor synthesis when it was demonstrated that tamoxifen, an oestrogen antagonist, could suppress the production of PNA receptors in animal mammary tissues even in the presence of 17β-oestradiol[93,105]. An 85% response to hormone therapy was observed in PNA receptor positive mammary carcinoma compared to a 24% response to PNA receptor negative tumors[93,105]. Oestrogen treatments which resulted in the inhibition of neoplastic growth were found to be associated with marked secretory changes and oestrogens have been seen to induce the biosynthesis and secretion of large amounts of PNA receptors. Thus, the synthesis of large amounts of PNA receptors could indicate that neoplastic tissues are undergoing transformation from a functionally undifferentiated state with high proliferative activity to a highly differentiated state with inhibition of mitotic activity which at times resembled a lactating mammary gland[105]. With these observations and results, PNA could be used to make the distinction between hormone sensitive and non-sensitive tumors and thereby giving the advantage over hormone receptor analysis in that the procedure could be performed on fixed tissue and on very small amounts of tumor materials especially metastases[93,105].

Carcinomas of the gastrointestinal tract

Tissue sections of carcinomas of the gastrointestinal tract usually show the expression of peanut lectin binding sites while normal mucosal surfaces have been found to bear very little if any PNA binding sites[133,134]. At the cellular level, a variety

of cells isolated from the gastric mucosa and the intestinal mucosa demonstrated PNA reactive sites[133]. The Isthmus cells of the gastric mucosa and the epithelial cells of the intestinal mucosa revealed high amounts of PNA binding sites which were almost always localized at the region of the golgi apparatus and this binding reaction has been proposed to be possibly due to the early phase of glycoprotein synthesis which was most apparent in surface epithelial cells[133]. The cells of the cardiac, antral (gastric mucosa) and Brunner glands (intestinal mucosa) although showing strong PNA binding sites localized to the golgi region in some of the tubules, also demonstrated PNA binding activity in the cytoplasmic granules in some of the other tubules thus giving a mosiac-like binding pattern with PNA[133]. This observation of binding pattern was probably due to differences in the functional and secretional stages of the gland cells[133]. The invaginated membrane systems of the parietal cells showed only selective binding to the peanut lectin while the luminal membrane surfaces of the chief cells were reported to exhibit a very intense positive reaction with PNA. Goblet cells of the small intestine revealed PNA binding sites that were both exposed and partly covered with neuraminic acid while the goblet cells of the large bowel were negative for PNA receptors[133].

Surface mucus cells of the normal colon were found to be devoid of PNA receptors pre- and post-treatment of the tissue sections with neuraminidase[133,134]. Some PNA binding activity was observed with the glandular epithelial of the antral mucosa of the stomach while weak binding with PNA was noted on the glandular epithelial of the body mucosa of the stomach only after neuraminidase treatment. Other tissue sections of the human gastrointestinal mucosa such as those isolated from the various parts of the colon, ileum, stomach were found to be PNA receptor negative even after neuraminidase treatment[85,134]. However, mucinous cells as well as the mucus within glandular structures have been reported to react positively with PNA[134].

In conditions of cancer or inflammation of the gastric mucosa and the transitional zones near the ulcus, hyperplastic alterations of the gastric mucosa such as

in surface mucus cells and isthmus cells are often seen. An increased binding acitvity of PNA in the perinuclear or the golgi region of the cells in the isthmus was always seen to accompany the elongation of the parts of the glands due to the hyperplastic conditions[133]. In processes of gastric adenocarcinoma, the extracellular mucus and the cell border of the mucus had high binding affinity for PNA after the tissue sections were pretreated with neuraminidase and approximately 50% of these tissue sections were PNA receptor positive with no enzymatic pretreatment compared to the absence of PNA receptors found in the normal mucosa tissue sections with and without neuraminidase treatment[133]. In large bowel carcinoma, PNA binding sites were localized to the region of the glycocalyx and intracytoplasmically in the apical portion of the cell while in normal cases PNA binding was only seen on the supranuclear portion of the cells[135]. In patients whose tumors were found to express the T-antigen, the benign epithelium in the non-transitional zone did not express PNA binding sites in 66% of the cases studied[135]. A possible explanation for this observation of the increased amounts of T antigen in cancer patients compared to normal subjects is the reduced amounts of glycosyltransferases in tumors which results in the incomplete biosynthesis of the MN blood group system thus resulting in the exposure of the precursor structure, the T antigen. Although 79% of the rectosigmoid adenocarcinoma cases were found to express the T antigen compared to the 40% found in normal epithelium, a difference of pattern distribution of PNA binding sites were seen[135] and this same difference of cellular localization of PNA binding sites have also been reported to occur in breast cancer where it was seen that breast carcinomas had PNA binding sites localized to the glycocalyx regions as well as intracytoplasmically while normal breast epithelium were found to express PNA binding sites only in the glycocalyx region[94,130]. In both cases the more pooly differentiated cancers were often found to be cryptic T antigen positive[94,130,135].

Tissue sections of diagnosed colonic hyperplastic polyps have been reported to give a peculiar binding pattern with PNA that was not affected by neuraminidase treatment. It was shown that the golgi region in the cytoplasm of the epithelial cells have granules with high binding affinity for PNA while the intracellular mucus located in the apical portion of the cells and the goblet cells were always PNA receptor negative[15]. In view of all the differences found between normal and abnormal conditions of the gastrointestinal tract with regards to PNA affinity, it would appear that PNA would have a potential role in the differential diagnosis of certain gastrointestinal tract disorders.

Peanut lectin in kidneys

Recently, the specific binding affinity of the peanut lectin was employed to investigate the potential utility of PNA in studying kidney anatomy and physiology with hopes of using the lectin in diagnosing kidney disorders. Histochemical studies with the peanut lectin on frozen kidney sections of various animal species resulted in the observation that the PNA binding pattern was species-specific and not strain specific[108].

In ten different strains of mice studied, the Bowmann's capsule, proximal tubules and distal tubules were PNA receptor positive[108] and with further investigations, receptors for PNA were also found in the membrane and cytoplasm of the collecting ducts[10]. In rats, the convoluted proximal and distal tubules and the collecting ducts were found to be PNA receptor positive while the glomerulus, proximal straight tubule cortex and the proximal straight tubule medulla were unreactive with PNA[108,132]. The proximal tubules in the rat were found to react with PNA stronger in the brush border than in the cytoplasm or in the basement membrane. In rats, the peanut lectin binding intensity was observed to decrease from the beginning to the end of the tubule with the end of the proximal convolution having hardly any PNA binding activity[132]. PNA binding was seen along the luminal membrane of the thick ascending limb medulla and cortex with the binding activity localizing at the apical rim of the cells. This

binding of PNA was seen to continue to the distal convoluted tubule on the luminal membrane and some activity was also seen in the cytoplasm in the later segments of the tubule. Both cell types, principal and the intercalated cells, in the collecting ducts were PNA receptor positive[133]. With rabbits, no PNA receptor sites were found in the glomerulus and in the proximal tubules[108]. PNA binding sites in the rabbits were seen localized to the luminal membrane of the intercalated cells in the connecting tubules and collecting ducts and some recptor sites were also found in the thin descending limb of the loop of Henle found in the inner stripe and the inner zone of the medulla[130].

In normal human kidney tissue sections, PNA receptors were seen to occur in the thin limbs, distal convoluted tubules, collecting ducts and the glomeruli while no receptors were found in the proximal tubules[108,127]. These binding sites occurred in a free as well as in a cryptic manner covered with neuraminic acid and the sites were found mainly localized to the luminal surface of the epithelial cells[127,128]. It was found that with neuraminidase treatment of the normal human kidney tissue sections an increase in the number of PNA receptors were obtained but this was not so with the epithelial cells of the proximal convoluted tubules as these cells were still found devoid of PNA receptor sites even after neuraminidase treatment[127]. In cases of hypernephromas, a wide range of distribution and number of PNA receptor sites occurred within the cytoplasm and especially on the tumor's cell membrane. Some hypernephromas resulted in the normally PNA receptor negative proximal convoluted tubules binding PNA[127]. In haemolytic-uraemic syndrome caused by neuraminidase from pneumococcal infections, the kidney tissue sections examined were found to bind PNA along the capillary loops of the glomerula while in patients with haemolytic-uraemic syndrome but without pneumococcal infections these areas showed no PNA binding sites at all[128]. The glomerulus of the patients with haemolytic-uraemic syndrome from pneumococcal infections exhibited high PNA binding activity on the podocytes and to a lesser extent on the endothelial cells[112,128] due to the *in vivo* exposure of these sites to the neuraminidase from the pneumococcal pneumonia while the distribution of these receptors in the normal glomeruli was only seen after treatment of the

tissue sections with neuraminidase *in vitro*[111]. The histochemical use of lectins has thus been reported to be a valuable tool in the differentiation of glomerular and tubular structures during embryonic development as well as in dystogenetic kidney tumors[127].

Treatment of all the kidney tissue sections of all the different animal species and strains with neuraminidase resulted in the finding of PNA binding sites in the glomerulus of all the animals tested thus implying the importance to the animals' glomerulus the presence of a sugar configuration in which the subterminal galatose containing PNA binding sites are covered with neuraminic acid[109]. The glycosylation of the nephrons is reported as species specific but not strain specific, thus indicating that the results obtained in the use of lectins in tissue studies cannot be directly applied from one animal species to another, even with closely related species[108].

Peanut lectin affinity in the skin

Various concentrations of peanut lectin receptors have been identified in the different layers of the normal human skin. The horny layer, granular layer and prickle cell layer were found highly positive for PNA binding sites while the basal layer, basement membrane and stromal fibers exhibited slight if any PNA binding activity upon incubation of the tissue sections with labeled PNA[85,89]. The peanut lectin has been found not to react as prominently in neoplastic epithelia compared to normal epidermis[89]. In squamous cell carcinoma, it was found that the binding affinity for the tumor cells varied accordingly to the degree of keratinization[85] with fully keratinized cells not reacting with PNA and tumor cells undergoing the process of keratinization exhibiting the most intense PNA binding activity[89].

Biodistribution of the peanut lectin

The peanut lectin with its demonstrated binding affinity for the T antigenic carbohydrate structure *in vitro* has been further investigated as a potential tumor localizing agent carrying a radionuclide for the possible radioimaging of T antigen bearing tumors. It was

reported that with the use of I-125 labeled PNA in animals bearing a T antigen tumor, good tumor localization with rapid blood background clearance was obtained thus resulting in clear serial scintigraphic images of the tumors by 24 hours post injection of the radiolabeled PNA[45,46,76]. No background subtraction was found to be necessary to obtain a clear delineation of the tumor. Biodistribution studies with the labeled PNA in the tumor bearing animals supported scintigraphic observation that PNA was indeed a tumor localizing agent with T antigen bearing tumors and that the affinity of the lectin towards the tumor *in vivo* was specific in nature[45,46,76].

In a recent human trial, peanut lectin radiolabeled with Iodine-131 has been intravenously administered to patients with known metastatic [illegible]rs of the colon, breast or lungs. Out of the 8 patients tested, 6 were found to have significantly higher amounts of circulating T-antigen in the serum[92]. However, only two out of the eight patients showed positive scintigraphic images at certain known anatomical metastatic sites. Another two patients with malignant pleural effusions that were adjacent to the carcinoma were found to take up PNA at the tumor site[92]. None of the patients showed any evidence of toxicity or reactions to ^{131}I-labeled PNA. Rapid blood clearance of ^{131}I-PNA was observed with 82.3 ± 5.3% (S.D) of the administered radioactivity recovered in the urine 24 hour post-injection[92].

E. Monoclonal antibodies

Introduction

The use of antibodies raised against a normal tissue and labeled with a radioisotope for subsequent scintigraphic imaging of the tissue localization of such a radiolabeled agent was first introduced in 1948 by Pressman and Keighley[136]. This approach was applied to the radioimmunodetection of tumors and in 1957 saw the use of radioiodinated antibodies that had been raised against certain animal tumors in the external imaging of the tumors[17,113]. Bale *et al* in 1960 demonstrated that such tumor localizing antibodies could be used to carry therapeutic

doses of radioactivity to irradiate the tumors[139]. Since then, investigation of antibodies for the radioimmunodetection or for the radioimmunotherapy of tumors has been of continual interest[13,140-142] such as the use of radiolabeled antibodies in the radioimmunodetection of tumors containing the alphfetoprotein[142] and in human colon tumors expressing the carcinoembryonic antigens[143]. It was soon discovered that the production of antibodies is always plagued by the uncertainties that resulted from the unpredictability and heterogeneity of the immune response of an immunized animal, and it was further shown that the affinity and quality of the antibody produced often varies from animal to animal and from the different times the animal was bled post-immunization[13,143,144]. Even the most potent and specific antisera available have often been found to contain antibodies of different classes and subclasses with different affinities for the same determinant and with different subspecificities and cross reactivities which have often resulted in problems when trying to compare results obtained with different serologic reagents, and also makes some antisera rather limited in availability and makes careful quality control essential even in routine serologic tests[13,143,144].

Renewed interest in the use of antibodies in the radioimmunodiagnosis and therapy of cancer has been generated by the hybridization technique reported in 1975 by Kohler and Milstein[9] which makes it possible to obtain a monoclonal antibody with a specific affinity that recognizes a specific antigenic determinant and hence minimizes, and in some cases eliminates the need to evoke such often used terms such as "interference", "heterogeneity", "cross-rectivity" and "non-specific binding" when discussing antibody functionality[146,147]. Monoclonal antibodies recognize only one determinant, and are of one class being derived from a single cell[146,148], and with this exquisite specificity and current availability, monoclonal antibodies are contributing significantly to advances in many research and diagnostic fields. Monoclonal antibodies are beginning to displace the use of polyclonal antisera in many facets of science[13,143,147] such as radioimmunoassays, enzyme immunometric assays, precipitin assays, immunofluorescence and immunohistochemical staining. Monoclonal antibodies have the added advantage over polyclonal antiserum in that the immunogen used to immunize the animal need

not be high[illegible]d since any unwanted activity in monoclonal antibodies can be eliminated by selecting against the antibodies responsible during the screening phase, hence impure immunogens may also be used to immunize animals in the prodution of monoclonal antibodies[143,147]. In general, a monoclonal antibody can be expected to present most of the desirable characteristics of a polyclonal mixture with few, if any of the undesirable traits. The limiting factor to obtaining the "perfect" antibody would most likely be the ability to devise screening methods[150-152] without sacrificing speed or efficiency.

Theory in the prodution of monoclonal antibodies

The immune response to an immunogenic stimulus consists of many single antibody producing cells[153], each secreting antibody independent of the others resulting in a total humoral response which is one of the many cells secreting different immunoglobulins at different times and rates[146]. Antibody producing cells from immunized animals have a very short life as they cannot grow *in vitro*[146,154] but this problem was overcome by the technique developed by Kohler and Milstein[9] that immortalizes antibody secreting cells by fusing them to myeloma cells, malignant cells that secrete immunoglobulin protein in an irregular and uncontrolled manner[146,155]. Gene complementation exists when fusion is made with antibody producing B cells, which are capable of producing immunoglobulins of defined specificity, and myeloma cells, which are capable of indefinite growth in culture resulting in hybrid cells which by inheritance from both parental lines now have the capacity for unlimited growth and the ability to secrete large amounts of specific immunoglobulins[146,147,155]. Non-immunoglobulin secreting myelomas are also available for fusion which yields a hybrid that secretes no myeloma protein but only that coded for by the spleen (B lymphocytes) cells material[146,155].

Technology of hybridization

The technique of cell hybridization was first demonstrated in 1965[157] but modern hybridoma work began in only in 1970[158] by Periman. Schwader and Cohen[159] in 1973 managed to isolate a hybrid that secreted both human and mouse

immunoglobulins when they fused mouse myeloma cells to human peripheral lymphocytes, whilst Cotton and Milstein[156] around the same time reported a similar phenomenon in the production of rat and mouse immunoglobulin from a mouse-rat myeloma hybrid. It was, however, Kohler and Milstein[9] who developed the technique of immortalizing individual clones of antibody secreting cells by fusing these cells to cultured mouse myeloma cells. The myeloma cell line P3-X63-Ag-8 first used[9] for the derivation of anti-sheep red blood cells is an 8-azaguanine resisitant line which lacks the enzyme hypoxanthine guanine ribosyl transferase (HGPRT), and also are unable to propagate under certain conditions. Most of the commonly used myeloma lines that are selected according to the method devised by Littlefield[160] are mutants which are lacking the enzymes HGPRT or thymidine kinase but are able to grow in the presence of azaguanine or bromodeoxyuridine[146,154].

The spleen cells that are used for fusion are obtained by aseptically dissecting the spleen from an immunized animal and isolating the cells from the cortex of the spleen. The immunization protocol is very much the same as that for polyclonal antibodies with most priming schedules consisting of subcutaneous or intraperitoneal injections followed by a secondary booster shot intravenously a few weeks later. The prepared spleen cells are then fused with the mutant myeloma cells, and the probability of fusion was found to increase greatly when the two cell types were exposed to fusing agents such as ultra-violet inactivated Sendai virus, lysolecithin or polyethylene glycol[155]. After fusion the cells are placed in a medium containing hypoxanthine, aminopterin and thymidine (HAT). Here, the cells that are deficient in HGPRT cannot utilize hypoxanthine and since aminopterin, a folic acid antagonist, is present, the *de novo* pathway by which cells effectively synthesize nucleotides is blocked. Hence, these cells that are deficient in HGPRT will not survive in the HAT medium. As well, all the selected myeloma mutants and myeloma x myeloma hybrids which all have their *de novo* pathway blocked by the presence of aminopterin will not survive. Hybrids that are

formed between the immunized spleen cells which contain the wild type salvage pathway enzymes and the mutant myeloma cells will be the only type of cells that can be found actively growing in the selection medium. Normal spleen cells that remain unfused have a limited life span *in vitro* and after a short period in culture, it could be safely assumed that any unfused spleen cells are absent.

Several techniques exist for the production of monoclonal antibodies although the original method used by Kohler and Milstein[9] as described briefly above is the most widely used[161]. Other fusion methods have been developed and used such as the fusion between human B lymphocytes and murine or other animal myeloma cell lines[157,162,163], human x human hybrids[164,165] and the Epstein-Barr virus transformation of B lymphocytes[166,167]. In most monoclonal antibody production using murine myeloma cell lines, the myeloma cells are usually developed from BALB/c plasmacytoma MOPC-21 which had been adapted to *in vitro* culture growth and this line as been renamed P3K[168]. The original line used by Kohler and Milstein[9] was found to secrete both the heavy and light chains of the original MOPC-21 tumor, but a variant of this line P3-NS1-Ag4 which is currently the most extensively used murine myeloma line for hybridoma production synthesizes but does not secrete the light chain and does not synthesize the heavy chain at all[169]. Other myeloma lines that are presently in use which do not synthesize any immunoglobulins are the X63-Ag8-653[170] and the Sp2/0-Ag-14[171].

Selection of successful hybrids

The immune response of an animal to an immunogen usually results in the stimulation of a widely heterogeneous population of cells, each secreting different antibody molecules[13,143,144] with the hybrid myelomas representing a cross section of this heterogeneous population[146,148,149]. Among the hybrid myelomas produced, only some will be secreting the antibodies exhibiting the desired properties. The fusion process of the immune spleen cells to the myeloma cells is a random event and there is no certainty that a desired hybridoma secreting antibody of the specificity sought will

be obtained, although it has been noted that on the average one hybrid is generated per 2 X 10^5 spleen cells and hence one can expect approximately five hybridomas producing the antibodies of interest from an average single mouse spleen[143,144,154]. Although only about 5% of the spleen cells actively secrete immunoglobulins of any description, successful hybrid clones have been seen to be much enriched in antibody production. It has been suggested that the enrichment factor may come from several possible causes such as the preferential recovery of hybrids involving actively dividing cells or an enrichment factor brought about by phenotypic complementation[154]. It has also been further observed that B cells that do not secrete but express the antibody have started secreting after fusion with the actively secreting myeloma cells which confer the highly secretive phenotype to the B cells[172]. This, then, results in hyperimmune spleens which have non-actively secreting antibody cells which start secreting antibodies with the hybrid derivatives produced[172]. After fusion and selection in HAT medium, successful hybrids are usually screened for immunoglobulin production[150-152]. When hybridomas are found which produce the antibody against the immunogen or antigen selected, the hybridoma is then cloned and the positive clones found to produce the desired monoclonal antibody may then be further propagated in culture, in appropriately primed animals, or kept frozen in storage[155].

Uses of monoclonal antibodies

The use of monoclonal antibodies in the field of science is very broad and even within the confines of clinical applications, their uses can only be considered under the broad headings of diagnosis, treatment and prophylaxis[164]. Monoclonal antibodies are fast replacing polyclonal antisera in many *in vitro* uses such as radioimmunoassays, enzyme immunometric assays, precipitin assays, immunofluorescence and immunohistochemical staining[13,143,144,147-152,161]. There are numerous reports also on the *in vivo* uses of monoclonal antibodies in the radio-immunodetection of tumors, immunotherapy of tumors by direct interaction or with either a

radionuclide or toxin conjugate[142,173-176]. The following discussion on the subject of monoclonal antibodies here will be confined to some of the diagnostic and therapeutic applications of monoclonal antibodies in cancer.

The availability of monoclonal antibodies of both human and non-human origin that are directed against cell membrane antigens which are phenotypically distinctive for various specific types of human neoplasms would be highly advantageous to immunodiagnostic and immunotherapeutic applications, and may further have prognostic significance in cancer management. There are, however, inherent problems in distinguishing phenotypically distinctive neoplasm antigens or tumor associated antigens. Surface antigens may be anomalously expressed on tumor cell suface which are not found on the normal cell from which it was originally derived but these same antigens may also be produced and expressed by other normal cells[16,176]. Tumors that result from the clonal expansion of single cells may magnify the expression of differentiation markers that are characteristic of the normal cells from which they are derived, and many of the tumor cell surface associated antigens are found to be actually normal molecules that are inappropriately expressed[16,176]. However, once tumor markers are identified, these unique markers would have considerable significance for the diagnosis, localization, monitoring and treatment of tumors in patients[16,176].

Radiolabeled monoclonal antibodies in cancer management presently offer the potential for 1) the detection, location and determination of the extent of disease upon the initial discovery of the cancer, 2) the characterization of the cell type of the neoplasm, 3) the follow-up of the progress of the residual lesion after initial treatment, 4) the early identification of any recurrence or metastasis, 5) the assessment of the effects of treatment, 6) the treatment of the tumor by means of systemic radiation therapy, 7) the prophylaxis against metastatic seeding in the course of or in association with other means of treatment and 8) the targetting of agents to tumors[174,176].

Diagnostic uses of monoclonal antibodies

There are presently three basic diagnostic approaches to the detection of cancer with the use of monoclonal antibodies and they are *in vitro* serologic tests, *in vitro* tissue analysis and *in vivo* radioimmunoimaging studies. The use of monoclonal antibodies in the serologic diagnosis of malignant neoplasms has been found to be promising and a growing interest in this area of immunologic detection is seen, but unfortunately, the diagnostic use of this method is limited to tumor antigens that are shed or released into the circulation. However, there are many surface membrane glycoproteins and glycolipids which are tumor associated that are highly antigenic and shed into the circulation. Thus, many of the monoclonal antibodies which are raised against such antigens may offer utility for this in the method of serodiagnosis. There are, at present, monoclonal antibodies being used to monitor certain types of malignant neoplasms such as colorectal, gastric and pancreatic carcinoma[110,114] in human patients by methods of serodiagnosis. The *in vitro* immunocytochemical analysis in tissue sections by monoclonal antibodies has by far the widest application in the diagnosis of cancer. Monoclonal antibodies are now being used for certain applications of immunopathologic techniques to cancer diagnosis where once heteroantisera were used. Monoclonal antibodies allow the identification of new prognostically significant disease sub-groups. This would allow immunopathologic dissection of closely related antigen families using monoclonal antibodies toward slightly different antigens[161,182]. Monoclonal antibodies that are elicited to surface antigens on a wide variety of normal and neoplastic cell types are being utilized with increasing frequency in the diagnosis and classification of tumors, identification of the tissue [illegible] malignant neoplasms and in some cases, to monitor the course of disease [illegible] cological and immunomorphological methods using monoclonal antibodies can be also used to study the expression of antigens or particular determinants in tissues, cell lines or even single cells[183]. Two major assay methods, immunoperoxidase and immunofluorescence staining, are now

being used extensively with monoclonal antibodies in the study of lymphomas and solid tumors[178-181].

The single most important application of monoclonal antibodies in the clinical diagnosis of cancer is perhaps in the radioimmunoimaging of tumors with radiolabeled antibodies[164]. A basic prerequisite for this application is that the monoclonal antibodies used should be of high yield in production and ideally react specifically with tumor associated antigens[4]. In reality, most of the monoclonal antibodies produced so far have only reacted preferentially with neoplastic cells but even this has been considered acceptable providing that the binding to normal cells does not in itself induce any adverse reactions. Potential antigenic sites on tumor cells have been observed to be abundant but many of these sites may not be fully available to the injected antibody[4] and hence, the accessibility of antibody to the antigenic sites on the cancer cell membrane may be a limiting factor to successful radioimmunoimaging. The delivery of the antibody to the antigen of interest *in vivo* has been found to require 1) adequate and homogeneous blood supply to the tumor, 2) movement of antibody from the intravascular space through capillary pores into the interstitial fluid and 3) movement of the antibody through the interstitial fluid in search of the antigen[8,13,15,186]. Other features of the tumor such as density, vascularity, vascular permeability, size and presence of shed antigens in the circulation will also influence the uptake of the antibody by the tumor. It has been demonstrated that radiolabeled monoclonal antibodies to tumor associated antigens do clearly localize within solid tumors in animals and humans[145,187,188]. The radioimmunodetection of tumors with labeled monoclonal antibodies would be most useful in the detection of occult metastases after the induction of a clinical remission, detection of potentially resectable lesions at an early stage, and to detect clinically undetectable tumors or metastases which are not identifiable with current techniques[190]. The use of labeled monoclonal antibodies have been found in some cases to be superior to conventional polyclonal antisera in the

radioimmunodetection of tumors[185] but there are some investigators who have found otherwise[191].

There are potential problems associated with the use of monoclonal antibodies *in vivo* and one of their main limitations is that monoclonal antibodies have been found to modulate surface membrane antigens of the tumor cells and hence, with this possible antigenic modulation occurring the tumor may then be rendered resistant to further use of the same antibody in diagnosis, therapy or monitoring the efficacy of treatment of the tumor[190]. Other problems that may arise are that these monoclonal antibodies may induce serum sickness or any unwanted side effects in the patient as they are foreign to the patient. False negative results have been reported to occur with the use of monoclonal antibodies in the detection of cancer as these antibodies tend to bind more avidly to viable tissue than areas of necrosis or fibrosis[185], and poorly to areas of low grade malignancy[192]. False positives have also been seen with the use of these antibodies in lymphoscintigraphy in a variety of solid tumors[193].

Therapeutic applications

The basic concept in the role of antibodies in immunology is the destruction of pathogenic cells and it has long been the hope that antibodies directed against tumor cell surfaces could be exploited for therapy[194] as antibodies provide an appealing theoretical approach in cell destruction *in vivo* with regards to their specificity and cytotoxic effects[195]. The primary limitation on the use of antibodies as therapeutic agents has been their impurity and heterogeneity but renewed interest has since been generated due to the advent of monoclonal antibodies which are homogeneous, react with constant avidity to single antigenic determinants and can be isolated in quantities previously not obtainable by conventional methods[195,196]. Monoclonal antibodies of defined specificity which can be continuously produced by permanent cell lines using the hybridoma techniques provide an appealing interest in cancer immunotherapy because of their specificty and also because they could be conjugated to radionuclides,

drugs and toxins to treat the condition. There are potential problems associated with the use of monoclonal antibodies in therapy that have been demonstrated in both animal and in clinical trials such as:

1) the specificty of the antibody for tumor cells as monoclonal antibodies which have affinity for antigenic determinants that may be common to both benign and neoplastic cells would cause lysis of normal cells as well as the putative target cells upon administration[195] but, in animal studies using a monoclonal antibody against an antigen that was determined to be simultaneously expressed by both normal and leukemic cells it was found that the antibody used did prolong survival time and cured a significant proportion of the treated animals[196].

2) the presence of phenotypic heterogeneity of tumor cell surface antigen since studies have shown that phenotype heterogenetiy occurs not only within a tumor of a given patient but may also be found in tumors at different sites in that same patient and in the same tumor type in different patients. Therefore, the use of a single monoclonal antibody would seem insufficient but a mixture or a 'cocktail' of monoclonal antibodies which recognizes different antigenic determinants may thus provide an armamentarium against individual subsets of the entire tumor cell population[139,195-197].

3) the administration of a foreign protein i.e. monoclonal antibody could result in the stimulation of an immune response from the host which in turn may complex and deactivate the administered antibody or may even result in serum sickness and/or anaphylaxis[195,196]. This has been the case in most of the murine monoclonal antibodies used. The use of human x human hybridomas or the administration of a single bolus of the antibody and possibly the use of an immunosuppresive agent prior to the administration of the antibody may alleviate the problem[195,196].

4) the presence of free circulating blocking factors or shed surface antigens of neoplastic cells in sufficient concentration in the peripheral blood could bind to most of the infused monoclonal antibody and hence prevent the antibody from binding to the

neoplastic cells[16,195,196].

5) the phenomena of antigenic modulation as first described by Boyse and Old[191] which pose as an important mechanism of cell escape. Many of the monoclonal antibodies used have been reported to induce antigenic modulation but this phenomenon was found to be reversible. The process of cell surface antigenic modulation may come about by the internalization of the antigen-antibody complex, shedding of the complex into the external mileu or the steric rearrangement of the complex within the cell membrane[196,199,200].

6) the process of immunoselection that results in the outgrowth of tumor cells which express the antigen during therapy with monoclonal antibodies. This has been found to be irreversible and is a problem that is common to both immunotherapy as well as chemotherapy. The occurrence of this problem was found to depend upon the frequency of antigen-negative cells in the original cell population and also on the efficacy of the treatment on the sensitive (antigen-positive) population[196].

7) the discovery of the continued growth of antigen-positive cells even though the cells were coated with the antibody. This problem has been attributed to a failure in host mechanism to eradicate more than a certain number of antibody coated cells which is most probably due to a shortage of effector cells in that area[196,201].

The therapy of solid tumors would be less than ideal compared to leukemia as it is questionable as to whether the monoclonal antibody infused would be able to penetrate all of the tumor mass. Immunotherapy would be enhanced if used on a smaller tumor load or on a reduced tumor load via prior radiotherapy, chemotherapy, and/or surgery[195]. It might well be that adjuvant therapy of micro-metastasis with monoclonal antibody would be their most significant clinical application, and if successful would prevent many relapses and may even convert complete remissions into true cures[144]. It is the obvious goal of many investigators to use an antibody that recognizes a tumor specific antigen which can distinguish absolutely between tumor

cells and normal cells but it has been found increasingly doubtful that such antigens exist[196,202]. Instead, most of the defined tumor related antigens appear to be embryonic or differentiation antigens which are expressed simultaneously on certain subpopulations of normal cells, either during embryonic development or at a specific stage in the differentiation pathways of the cells of origin of the tumor. Hence, any monoclonal antibodies that are sufficiently cytotoxic to cells bearing the antigens would also kill many of the antigenically related normal embryonic or differentiating cells[164,176,196]. Most of the reported monoclonal antibodies which show specificity for human malignant cells such as melanoma[204,206], colorectal carcinomas[203] and lymphomas[207] have also been shown to react with normal cell types[176]. Most of the immunotherapy performed with some success is in the treatment of leukemias as the majority of these neoplastic cells are in the peripheral circulation[195,203]. There have been attempts made to treat solid tumors such as gastrointestinal tumors[201] and malignant tumors[209] but with rather limited success. The use of murine monoclonal antibodies as immunotherapeutic agents in humans has resulted in more than half of the patients exhibiting significant antibody responses against the murine immunoglobulin but there were few if any incidences of antibody responses that resulted in allergy, serum sickness, immune-complex disease or other evident toxicity even though the murine immunoglobulin material was prepared from the ascites fluid by mice bearing the hybridoma and the resultant final product found containing other serum proteins[196] and doses as high as 1500 mg have been administered[207]. There were a few limitations observed when using murine monoclonal antibody for immunotherpy in humans such as 1)a finite capacity to eliminate antibody coated cells with antibody dependent cellular cytotoxicity or the reticuloendothelial system and 2)immunogenecity of mouse antibody resulting in antimouse antibody[195,196]. Other treatment failures are such as described earlier.

Other methods of using monoclonal antibodies for the therapy of tumors which appear rather promising include the conjugation of anti-tumor monoclonal antibodies to α-particle emitting radionuclides such as Radium-224 or Astatine-211[210], to chemotherapeutic agents such as alkylating agents cyclophosphamide and adriamycin, or to the A chains of toxins such as ricin and diphtheria toxin[196,203]. Antibodies specific for tumor antigens have long been considered as possible carriers for antitumor agents and greater interest in this has been generated with the introduction of monoclonal antibodies with predefined specificities[9]. Conjugation of monoclonal antibodies with chemotherapeutic agents and bacterial plant toxins have been performed in many laboratories and these conjugates have been found to have preferential cytotoxicity to the target cells which react with the antibody component of the immunotoxin[1]. It is still, however, not established whether there exist human antigens that are sufficiently tumor specific to provide a target for therapy with antibody-toxin conjugate[203] as the antibody conjugate should localize in sufficient concentrations in the target tissue to provide an adequate concentration of the attached therapeutic agent[1]. There are a few considerations to be determined such as the percentage of the injected dose taken up by the tumor because any excess antibody-conjugate still remaining in circulation might result in non-specific interactions and subsequent damage to normal tissues. The exact location and means by which the antibody-conjugate are localized within the tumors should also be determined as the therapeutic-conjugate agents may need to be internalized by the tumor cells to elicit a cytotoxic response[1,203]. The treatment of solid tumors may pose more problems as the penetration of antibody into the tumor tissue is restricted by the extent of vascularization and extravasation within the tumor mass[1,203]. Hence, smaller antibodies such as immunoglobulin G or their fragments may be preferable for the delivery of drugs to targets outside the bloodstream as they should have optimal diffusion properties. Larger monoclonal antibodies such IgM which tend to be retained within the bloodstream due to their large molecular size may be the most

suitable class of immunoglobulins for attacking intravascular targets[9]. Clinical problems that could arise in the use of an antibody-toxin conjugate are that neutralizing antibodies could develop against the injected antibody and antigen shedding from of certain types of tumor cells may neutralize the antigen recognition of the conjugate before it reaches the target[203].

Human monoclonal antibodies

For the diagnostic and therapeutic applications of antibodies in a human system, it would be highly desirable to use human rather than mouse or any other rodent derived antibodies. The clinical application of xenoantibodies would be severly limited by the fact that they are foreign proteins[149,164]. Before a potential human monoclonal antibody could be used clinically, there are two major problems that must be solved[149]. The first is to obtain stable lines that will continue to secrete the desired human antibody over long periods of time[149,164], and the second is to be able to select and predetermine the specificity of the selected antibody. This poses a greater problem in the human than in the mouse as it is ethically impossible to immunize human donors against the vast majority of antigens[149,164].

There are, presently, three basic approaches to obtaining stable human antibody secreting cell lines, which are, 1) the production of mouse-human hybrids[149,164,212], 2) Epstein-Barr virus (EBV) transformation of human lymphocytes[149,216-220,166,223], and 3) the production of human-human hybrids[148,149,165,173,222].

Mouse-human hybrids

Early attempts of producing human monoclonal antibodies made use of the hybridization technique developed by Kohler and Milstein[9], fusing human lymphocytes with mouse myeloma lines used for the production of mouse monoclonal antibodies[172]. This technique has been questioned as a viable means of producing human monoclonal antibodies as it has been observed that the fusion product of mouse myeloma and human lymphocytes is genetically unstable resulting in the loss of human chromosomes

from the interspecies hybrid[165] over a short period of time[149].

However, several investigators have reported to be able to obtain stable heterohybrids secreting specific human monoclonal antibodies to antigens such as B-cell lymphomas[162], human mammary carcinoma[215], and lung tumor antigen[212], with some laboratories reporting the continued production of mouse-human hybrid monoclonal antibody cell lines over long periods of time[163]. Even though the resulting immunoglobulin produced from the mouse-human hybrid would be of human origin, the product would still have to be purified to remove any contaminating mouse protein which might be present either through specific secretion or through the breakdown of cells resulting in the release of cytoplasmic contents[163] before the product could be clinically useful.

Epstein-Barr virus transformation

The Epstein-Barr viral transformation of human lymphocytes has been described[216] as a promising technique to immortalize specific antibody producing lymphocytes, and this immortalization technique has been shown to retain certain characteristics of the B cell intactly so that the transformed plasma cell will continue to produce antibodies[148]. However, there have been cases reporting that EBV transformed cell lines have often been found to stop producing antibodies after a viable period[149]. The EBV is found to infect only EBV receptor positive cells[217], and thus restricts transformation to B lymphocytes. Epstein-Barr virus, the etiological agent of infectious mononucleosis[214] is also found to be associated with two human malignant diseases which are the African Burkitt's lymphoma[177] and nasopharyngeal carcinoma[138]. Infection of blood lymphocytes by EBV was found to induce polyclonal secretion of immunoglobulin[174]. The immunoglobulin production in EBV infected B lymphocytes is shown to be T lymphocyte independent although activated T lymphocytes could be involved as modulators of this response[211].

There are two ways in utilizing this transformation technique, which are 1) to isolate peripheral blood lymphocytes and culture them *in vitro* with the Epstein-Barr virus and the antigen of interest[223], and 2) to isolate and select peripheral blood lymphocytes for their response to an antigen[166]. This second method may be useful in raising antibodies to antigens which have reactive cells occurring naturally in the blood or have been induced by prior infection or deliberate immunization[141]. Another approach using the EBV transformation technique is to combine this method with the hybridoma technique. In this combination procedure, EBV transformed lymphocytes that are already producing antibodies are fused with a lymphoblastoid cell line[224]. This EBV-hybridoma technique has been found to produce a 4-fold to 8-fold more specific antibody than the EBV parent. The initial investigation of this method has reported a higher cloning efficiency with a stable production of antibodies at a higher rate than that of the EBV transformed lymphocytes[224].

Antibodies produced in this manner may be of use as research tools but their clinical use may be rather limited because of the potential danger to the patient of human-contained virus associated with infectious mononucleosis and such neoplasias as Burkitt's lymphomas[148,149] because studies have shown that some of the EBV immortalized cell lines as well as lymphoblastoid cell lines derived from normal seropositive donors could grow as malignant tumors upon heterotransplantation in nude mice[119]. Although the general use of this method is limited, continuous cell lines secreting antibodies against group A Streptococcal carbohydrate[218], antinitrophenol antibodies[219], and rheumatiod factors[220] are in current use.

Human-human hybrids

There is a considerable amount of interest in generating monoclonal antibodies from human cells[213] fusing human lymphocytes with human lymphoid cells[149,213] using the method described by Kohler and Milstein[9]. Such reagents should be better tolerated in the human system than are antibodies raised in other species such as rats or mice

since complications such as serum sickness or rapid elimination of foreign immunoglobulins would be minimized[147]. Lymphocytes obtained from cancer patients can be used to prepare the human-human hybridoma offering opportunities to study the immunology of relevant tumor associated antigens against which the lymphocyte donor has responded[147]. However, B cells in peripheral blood (the only reliable and easily obtainable source of human lymphocytes) comprises only about 30% of mononuclear cells and the majority of these are in resting non-activated state[221], and there is evidence shown that "activated" cells (those cells having undergone blast transformation) preferentially fuse to myeloma cells[222]. There are advanced tissue culture techniques now developed which make it possible to stimulate the B lymphocytes with an appropriate antigen *in vitro* and then fuse them to myeloma cells[165] implying that it may now be possible to obtain human monoclonal antibodies by *in vitro* priming with an antigen that cannot be used to innoculate a human[148]. A sparsity of potential myeloma fusion partners do exist as it has been found to be difficult to grow human myelomas in tissue culture[149], but there are reports of human lymphocyte fusions with various myeloma lines[165,173] and two human myeloma cell lines that have been found suitable for hybridoma production are drawing considerable attention[165].

F. TA3/Ha murine mammary adenocarcinoma

Origin of the tumor line

The TA3 tumor had originated as a spontaneous mammary adenocarcinoma in a female A/HeHa mouse in 1949 and had been continuously transplanted in the strain of origin[225]. It was converted to the ascites form on two separate occasions: The TA3/St ascites subline arose from the sixteenth solid passage[226] and the TA3/Ha ascites was derived from the 34th solid transfer generation[227]. The TA3/St ascites subline retained its transplantation specificity for strain A

mice, but acquired a hypotetraploid karyotype with two metacentric markers and a model chromosome number of sixty nine[226]. The TA3/Ha ascites subline, which in 1953 killed only strain A mice and had a diploid chromosome mode of forty[227], acquired one additional small acrocentric chromosome before 1956; it then became 100% allotransplantable, and developed immunoresisitance without any known selection pressure[221], to mice and was lethal for unconditioned rats[229] even at low tumor inocula[228].

Morphology

The TA3/Ha and TA3/St ascites cells are generally spherical when fixed in suspension[230] with the surface area of the TA3/Ha cell approximately 1.4 times smaller than the TA3/St cell[231]. The general internal morphology of both the TA3/Ha and TA3/St cells were found to be similar[230]. Both cell sublines were found to contain abundant virus particles which appeared to be concentrated in the region of the cytoplasm immediately surrounding the golgi membrane[230]. The TA3/Ha cell was seen to possess a regular topography with long and rigid microvilli distributed at regular intervals over the surface. The microvilli, rarely branched and fairly straight, were observed to be extended outward a distance equal to the cell diameter[230]. Intermicrovillous space on the TA3/Ha cells were generally smooth, and the surface lacks the membrane folds which are characteristic of the TA3/St cell[230]. A morphologically distinct and extensive cell surface coat reminiscent of the fuzz coat of the intestinal brush border was found on the TA3/Ha cell[230]. This coat most often appears as a network of fine elements about 5 nm in width extending 30-50 nm with individual filaments that may extend 200-400 nm from the outer membrane[230,232]. The surface coat of the TA3/Ha cell generally appears to be more extensive in the neighbourhood of high concentration of microvillous fragments[230,232] which appears to stablize these structures[232]. This heavily sialic coated[41] cell coat which covers the numerous microvilli characterizing the TA3/Ha cell[230] was shown to be glycoprotein in nature[233]. There is an estimated 5 mg. of this glycoprotein, now known as epiglycanin[230], per 1 x 10^9 TA3/Ha cells extending outward from the TA3/Ha cell membrane[234].

TA3/Ha cells have a mean electrophoretic mobility of 1.56 ± 0.010 μsec^{-1} $volt^{-1}$ cm, as compared to 1.27 ± 0.012 μsec^{-1} $volt^{-1}$ cm. for the TA3/St cells indicating a higher net negative charge for the TA3/Ha cells[228]. This higher net anionic charge of the TA3/Ha cell can be attributed to the presence of neuraminidase sensitive sialic acid[230]. There is approximately 580 ± 40 μg per 1 x 10^9 cells of the neuraminidase sensitive sialic acid on the TA3/Ha cell surface compared to approximately 270 ± 30 μg per 1 x 10^9 cells on the TA3/St cell surface[230]. Treatment of both cell sublines with neuraminidase will remove more than 90% of their cell surface sialic acid[230].

Biological characteristics

Growth characteristics

The TA3/Ha cells multiply earlier after explantation than the TA3/St ascites cells and will grow and remain as a suspension culture of single cells or small cell complexes[235]. Both these ascites sublines grow *in vivo* predominantly as single cell suspension with a tendency to form complexes of a few cells[221]. The TA3/Ha ascites cell will grow progressively in foreign species such as the rat and the hamster as well as in allogeneic mice[228]. Allotransplantability of the TA3/Ha ascites cells was found, with very few exceptions, to be restricted to the peritoneal cavity and not operative subcutaneously or intravenously[236]. Sequential passage of the TA3/Ha ascites cells subcutaneously in syngeneic mice has resulted in the adaptation of these cells to subcutaneous allogeneic growth[236]. It is believed that epiglycanin has a major role in permitting this allogeneic growth[236]. TA3/Ha cells which have been grown *in vitro* for more than ten weeks were unable to grow *in vivo* in allogeneic mice[237]. These cells were then found to have lost about 90% of their surface epiglycanin[237]. After repassage of these cells into syngeneic mice, they were found allotransplantable again and had also regained their surface epiglycanin[237].

Immunogenicity

The TA3/Ha ascites cells were found to possess approximately the same concentrations of histocompatability antigens (H-2) on their surface as the strain specific TA3/St ascites cells but have a lower concentration of accessible antigenic determinants for antibody binding capacity[235]. TA3/Ha cells, which produce both anti-H-2 [illegible] as well as sensitize lymphoid cells in allogeneic mice, were found to exhibit resistance *in vitro* to both humoral and cell-mediated cytotoxicity[235]. These TA3/Ha tumor cells are considered poor immunogens as it was found that even irradiated TA3/Ha cells are less immunogenic than the TA3/St cells in evoking either the humoral and/or cell-mediated immunity[228,235,238]. TA3/Ha cell resisitance was found not to be an absolute phenomenon since the cells can be killed by heterologous antibodies such as rabbit anti-mouse antisera in the presence of either guinea pig serum or rabbit serum as the source of complement but not with alloantisera[235]. The TA3/Ha cells were also observed not to possess intrinsic immunoresistance[2[illegible]

Transplantability

The TA3/Ha cells have been found to synthesize H-2 antigens in amounts similar to the TA3/St cells and these antigens reside in the membrane with only a small fraction exposed on the outer surface of the living TA3/Ha cells whereas all or most of these antigens are exposed on the surface of the TA3/St cells[239]. Ineffective immunogenecity rather than immune resisitance on the part of the TA3/Ha cells has been demonstrated as the major factor in the permissive growth of these tumor cells in histocompatible animals[238]. It has been suggested that a high molecular weight glycoprotein, epiglycanin, found only on the surface of the TA3/Ha cell, masked the histocompatibility antigen of the TA3/Ha cell and physically protected the antigenic sites from the binding of antibody, thus permitting the widespread allotransplantability and xenotransplantabiltiy of the TA3/Ha tumor cells[232,237,240]. It has also been suggested that the higher net negative charge of the TA3/Ha cells due to the sialic acid

content may facilitate the outgrowth of these cells in allogeneic mice[238] and it was further demonstrated that removal of sialic acid from these cells by process of neuraminidase treatment reduced the transplantability of the tumor in allogeneic mice[240] at low tumor inocula[232]. The removal of sialic acid from the TA3/Ha cell surface was found to expose new galactose terminated antigenic determinants on the cell surface which, in the presence of a factor in serum and complement, was cytotoxic to the neuraminidase treated TA3/Ha cells[232]. Sialic acid was found not to mask the surface histocompatability H-2 antigens on the TA3/Ha cell surface[232] as the absorption of anti-H-2 antibody by the TA3/Ha cell was not affected by the neuraminidase treatment which removed the surface sialic acid but not epiglycanin[241]. Allotransplantability in experiments with higher tumor inocula of TA3/Ha cells were found to be unaffected by the removal of sialic acid from the cell surface[240,241]. Allogeneic mice can be protected against the growth of the TA3/Ha cell with methods such as skin grafting[240], injection of either lyophilized of formaldehyde treated TA3/Ha ascites cells[242], pre-injection of TA3/St cells alone[238] or simultaneous injection of viable TA3/St and TA3/Ha cells[240].

Lectin binding specifities

TA3/Ha murine mammary adenocarcinoma tumor cells have been found to possess receptors for the lectins Phaseolus vulgaris, concanavalin A and *Vicia graminea*[42] but were not agglutinated by either one of these lectins[42,244]. Agglutination of the tumor cells by the lectins was determined to be uninhibited by the presence of sialic acid[42,244]. TA3/Ha tumor cells that have been treated with proteases however were agglutinable by concanavalin A and Phaseolus vulgaris. The strain specific TA3/St tumor cells have the same number of receptors for the lectins Phaseolus vulgaris and concanavalin A as the TA3/Ha tumor cells but were found to be agglutinated by these lectins without any prior treatment of the cells with enzymes[244]. Hence, differences do exist in the carbohydrate containing macromolecules on the cell surface of the two TA3

sublines, some of which are in a cryptic form on the surface of the immunoresistant TA3/Ha cells[244]. Both the TA3/Ha and TA3/St tumor cells can be agglutinated by the peanut lectin[45] and triticum vulgaris lectin[244] but the number of receptors for the peanut lectin on the TA3/St cells were considerably less than that found on the TA3/Ha cells[45]. The TA3/Ha tumor cells have also been found to absorb both human and animal anti-T antibodies as well as anti-N lectins such as the *Vicia graminea*[50].

TA3/Ha organ specific metastatic variants

Strain A/HeJ mice that had been injected with viable TA3/Ha tumor cells intravenously were found to result in distant tumor growths forming mostly to the lungs and to some peripheral or subcutaneous growth whereas with TA3/St tumor cells tumor growth was found mainly localized to the lungs[236]. Massive widespread tumor implantation in multiple organs including the liver, spleen, kidney, lymph nodes and peritoneum can be achieved following the intravenous injection of TA3/Ha tumor cells into the strain A/HeJ mice[236]. Epiglycanin has been hypothesized as the cause for the enhanced TA3/Ha metastatic spread of the tumor cell implantation beyond the lungs[236]. This high molecular weight glycoprotein that is present on the TA3/Ha adenocarcinoma tumor line has been determined to be present on the selected TA3/Ha metastatic tumor variant lines[236] and the TA3/Ha metastatic lung and liver variants have also been found to maintain a high expression of the Thomsen-Friedenreich antigen as well[45]. Selected TA3/Ha metastatic lung and liver variant tumor cells have been obtained with the *in vivo* and *in vitro* selection method described by Fidler[243] by Dr. B.M. Longenecker at the Department of Immunology, University of Alberta and the TA3 tumor cell lines used in the research project has been kindly provided by Dr. Longenecker. Further description of the TA3/Ha tumor cells is provided in the latter portion of the thesis.

Epiglycanin

The high molecular weight glycoprotein epiglycanin which has a molecular weight of approximately 500 000 daltons[43] is found to exist in an extended polypeptide configuration often longer than 400 nm in length and about 2.5 nm in width[245]. Epiglycanin, which is heavily sialylated, is present in large amounts on the TA3/Ha tumor cells. It has been approximated that there is about 4 mg of the glycoprotein per 1×10^9 tumor cells or about 5×10^6 molecules of epiglycanin per TA3/Ha cell[245]. Epiglycanin can be readily removed from the tumor cell surface by the action of trypsin or papain cleavage which has been found to yield fragments of the glycoprotein with an average molecular weight of approximately 200 000 daltons[245].

The composition of epiglycanin was found to be about 23% protein[232] and over 75% carbohydrates in the form of numerous short and longer side chains that are attached to serine and threonine residues of the protein backbone by O-glycosidic linkages to the reducing terminal sugar α-NAcGal[43,245]. In other words, there are more than 500 carbohydrate chains that are attached to a single polypeptide chain of about 1 300 amino acids[43]. Approximately 60% of the carbohydrate content in epiglycanin has been found to be present as the structure β-D-Gal(1→3)α-D-GalNAc bound by an O-glycosyl linkage to serine and threonine[246]. Cell surface epiglycanin is found to exist in two high molecular weight forms; a long chain and a short chain[247] and the distinction made between the long chains and the short chains rests solely upon the presence of a 2-acetamindo-2-deoxy-D glucose residue that is present in the long chains[43].

The dissacharide described as β-D-Gal(1→3)GalNAc which is a structure similar to that proposed for 60% of the carbohydrate content of epiglycanin has been reported to occur in many glycoproteins and many of these glycoproteins as well as the relation of the dissacharide to them has been very well reviewed in the literature by Vaith and Uhlenbruck[37]. This same dissacharide structure is also present on human erythrocyte glycoprotein that contains the M and N blood group activities[21,119] and has been also considered as the immunodeterminant structure of the Thomsen-Friedenreich antigen[27]. Epiglycanin was found to be as effective as

the most highly active N antigen preparations in specifically inhibiting the agglutination of human blood group NN erythrocytes by the anti-human blood group N specific extracts from the leguminous plant *Vicia graminea*[42]. Excellent inhibition of NN specific human erythrocytes by *Arachis hypogaea* in the hemagglutination by both neuraminidase treated and untreated epiglycanin was also observed[43]. Epiglycanin has also been reported to inhibit agglutination of the TA3/Ha and TA3/St cells by lectins[248] such as potato lectin, eel serum agglutinin and *Ricinia communis* agglutinin.

Viable TA3/Ha tumor cells have been found to shed the glycoprotein epiglycanin from the cell membrane into the ascites fluid and serum *in vivo*. *In vitro* analysis of the TA3/Ha tumor cells have found that epiglycanin is only shed by viable tumor cells as dead cells do not release epiglycanin[249]. There is a decrease in epiglycanin shedding from the surface of the TA3/Ha cells during growth of the tumor cells in suspension culture and a subsequent increase after repassage of the cells in syngeneic mice[233]. TA3/Ha cells which had lost their surface epiglycanin due to *in vitro* culture have been found to be unable to propagate in allogeneic mice but repassage of these cultured cells into syngeneic made them allogeneic again[237]. Hence, epiglycanin is important for the survival and growth of TA3/Ha tumor cells injected into allogeneic mice. Epiglycanin is also believed to be responsible for the ability of the TA3/Ha tumor subline to grow in allogeneic and xenogeneic hosts[240,242] by the process of physically masking or sterically hindering the H-2 histocompatability antigens hence, rendering these antigens relatively inaccesible for interaction[245]. An alternate explanation proposed was that epiglycanin molecules that are shed *in vivo* have a suppressive or blocking effect on alloantigen reactivity by the hosts[249].

Epiglycanin present on the TA3/Ha tumor cell surface and also shed into the serum has been indicated numerous times[232,237,240] as the factor responsible for the allotransplantabiltiy and xenotransplantabilty of the TA3/Ha tumor cells. It has also been implicated as the cause for the enhanced metastatic spread of the TA3/Ha tumor beyond the lungs[236].

G. Radioiodination

There are basically two major considerations when selecting a radionuclide for labeling a pharmaceutical for use in radioimaging procedures. They are a) the necessity of minimizing the radiation dose to the patient and b) the detection characteristics of present day nuclear medicine instrumentation[251]. The radionuclide of choice should, ideally, emit a monoenergetic gamma ray with energy between 100 - 300 keV to allow for sufficient tissue penetration with minimum scatter and maximum detection efficiency on available instruments[251,252]. The radionuclide should not emit any particulate radiation such as alpha and beta particles[253] and it should give a [redacted] of undesirable radiation such as those arising from Auger electrons, conversion electrons and low energy electromagnetic radiation following electron capture or internal conversion[252]. The radionuclide should have as short a physical half-life that is compatible to the physiological half-life of the phenomenon under study[251,252] as a lesser half-life may not allow sufficient clearance of the labeled product from the circulation with enough tumor accumulation at time of imaging and a longer physical half-life would result in unwanted radiation dose to other organs after peak tumor irradiation has been achieved[253]. The radionuclide should also be available easily, economically and in as pure a radionuclidic form as possible.

Isotopes of Iodine

The radioisotopes of iodine are considered to have the greatest synthetic versatility among the readily available gamma emitting radionuclides as they have been observed to label both hydrophlic and lipophilic compounds by a number of direct and indirect methods[254]. There are presently more than two dozen radioisotopes of iodine available[251], of which three ^{123}I, ^{125}I and ^{131}I are in common clinical use. Iodinated compounds have been found to deiodinate quite rapidly *in vivo*[254] and the free iodide is observed to be taken up usually by the thyroid, stomach and intestine, salivary glands and choroid plexus, with a large fraction filtered into the urine by the kidneys[251]. Iodinations have sometimes been observed to alter the original characteristics of

molecules and occasionally denature a macromolecule[254].

Iodine-131

Iodine-131, discovered in 1938[255,256], is available in large quantities quite inexpensively in the chemical form of sodium iodide or as ^{131}I labeled compounds. Iodine-131 which has a physical half-life of 8.04 days decays by beta emission and has a principle gamma ray emission of 364.6 keV with 82% abundance which is considered slightly higher than ideal[252] for scintigraphy. Iodine-131 also has two high energy photons of 637 keV and 722 keV emitted in 12% of the disintergrations[252] which will further decrease the resolution and image sensitivity of a scan. Iodine-131, commercially produced by either the thermal neutron activation of tellurium 130, ^{130}Te (n, γ) $^{131}Te \xrightarrow{\beta^-} {}^{131}I$ (Radiochemical Centre, Amersham, Bucks, England) or the irradiation of a ^{235}U-aluminium target in a neutron flux of 2×10^{14} neutrons/cm^2/sec, ^{235}U (n, f) ^{131}I (Oak Ridge National Lab, Oak Ridge, Tenn.). Iodine-131 is currently used in many medical institutions for thyroid function diagnosis, imaging and therapy[251].

Iodine-125

Iodine-125, discovered in 1946[259], is available economically[257] in almost 100% isotopic abundance[260] and has a physical half-life of 60 days which provides a shelf-life that is convenient for synthesis, storage and shipping[252]. ^{125}I decays entirely by electron capture with the emission of 28 to 35 keV photons which permit enhanced counting efficiency and scanning resolution[252] and [illegible] and increased tolerable isotope dosage[257]. Iodine-125 has been used extensively for *in vitro* studies due to its excellent detection efficiency[252] but not as frequent in *in vivo* studies as the 28 - 35 keV photons are readily attenuated by overlying tissues[258]. However, many investigators have considered I-125 to be advantageous over I-131 in numerous selected *in vivo* studies[261,262].

Iodine-125 is economically produced by the neutron bombardment of xenon-124, ^{124}Xe (n, γ) ^{125}Xe [illegible] giving relatively pure amounts of iodine-125[243] and this is the present commercial mode of preparation by the Atomic Energy of Canada Ltd., Chalk River, Canada whereby xenon-124 is irradiated at a neutron flux of 2.4 x 10^{14} neutrons/cm^2/sec for 60 days which produces 0.32 Curies of iodine-125 per gram of xenon-124[250,251].

Iodine-123

Iodine-123 has been commercially available since 1972 and is gaining widespread use[251]. Iodine-123 has a half-life of 13.3 hours and decays solely by electron capture[252] with a principle emission of 159 keV photons at 82% abundance[251]. Iodine-123 has been considered to be an ideal radioimaging agent[252] as it gives excellent scintigraphic images with good resolution while minimizing the radiation dose to the patient[253], but its physical half-life may be too short for some studies[257]. Available ^{123}I has been usually found to be contaminated with a longer lived radionuclide, iodine-124, which increases the radiation dose to the patient, decreases image resolution and limits its shelf-life[251].

There are at present many routine methods of producing iodine-123 utilizing direct as well as indirect nuclear reactions and the various methods are well reviewed in the literature[264]. The ^{124}Te (p, 2n) ^{123}I direct nuclear reaction using a small cyclotron has been considered as one of the more promising methods of ^{123}I production but the use of enriched tellurium-124 targets limits the availability and increases the cost of production[264,251]. The indirect methods of obtaining iodine-123 are from the decay of xenon-123 by the generator method $^{123}Xe \xrightarrow{\beta^+, EC} {}^{123}I$[264,264]. These indirect methods offer some advantages such as being able to obtain a higher radionuclidic purity of iodine-123 due to the separation of xenon-123 and the use of inexpensive naturally occurring target materials (iodine, lanthanium or cesium) for the production of xenon-123[250,264].

Protein radioiodination

Direct methods

The radioiodination of protein involves the incorporation of iodine atoms mainly into tyrosine and occasionally into histidine, tryptophan or sulphydryl groups of the protein, and the degree of iodination depends on the microenviroment of these side chains in the protein[254,260]. Radioiodine has to be in its active or oxidized form to be active for labelling. Oxidizing agents oxidize the iodide to iodine which in turn reacts with water to form the active species of iodine, H_2O^+I, known as the hydrated iodinium ion[254].

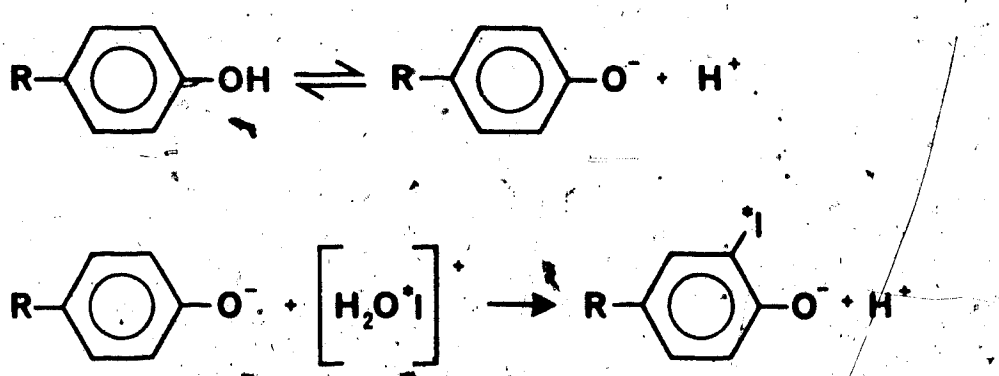


Figure 1: Iodination of tyrosine moieties

Many methods are available for iodinating proteins, each having their advantages and disadvantages. Hence, depending on the characteristics of the protein and the nature of the enviroment, a particular labeling method may be more suitable over the others in maximizing the maintenance of characteristics and properties of the protein. Below is a short and general description of several commonly used direct methods of radioiodination.

Iodine monochloride

Iodine monochloride which is highly stable in aqueous solutions has a theoretical high availability of iodine for iodination of proteins and this method has been used extensively for protein radioiodination resulting in high specific activity[271] and a high labeling efficiency of approximately 50% - 60%[265]. Iodine monochloride that has been equilibrated with radioiodide is reacted with the protein and mixed well for about one minute after which the reaction is terminated by the addition of excess sodium metabisulfite to reduce the free unreacted radioiodine to radioiodide. The radioiodide is then removed from the reaction vessel by conventional methods[254,260]. Iodination of tyrosine residues is preferred at lower pH values as iodination of histidine residues will result at a pH of 7[254].

This method has the advantage in that electrophilic substitution of iodine into the protein can be precisely controlled, but suffers the disadvantages in that the protein is exposed to potentially harmful radioiodide solutions and stable iodide is also incorporated into the protein resulting in lower specific activity[254,260,265].

Chloramine-T

The chloramine-T method[266] is a widely used method for radioiodinating a variety of proteins to high specific radioactivities[260,265]. Chloramine-T, the sodium salt of N-monochloro-p-toluene sulfonamide, is a mild oxidizing agent that slowly liberates hypochlorous acid in aqueous solution and oxidizes radioiodide to active radioiodinium, which is incorporated into the tyrosine residue of proteins[254,265]. The reaction is stopped by the addition of sodium metabisulfite which reduces radioiodine back to radioiodide and also stops the oxidation[254,265]. Free radioiodide can then be removed from the iodinated protein. The chloramine-T method has an optimal labeling efficiency at approximately pH 7 as the labeling efficiency is reduced at higher or lower pH. Above pH 8.5, iodine substitution into the imidazole ring of histidine residue is preferred[254,260]. This method of labeling is technically simple and rapid to perform with a high degree of

iodine incorporation into the proteins usually obtained but loss of activity and aggregation of proteins have been reported to occur with the use of this method[254,260].

Hypochlorite oxidation

An aqueous solution of sodium hypochlorite is substituted for chloramine-T in this radioiodination method which has sometimes been found to be better than using the chloramine-T method in preserving the immunoreactivity and biological activity of the protein[260].

Chlorine oxidation

Chlorine gas which is prepared in a closed vessel by the addition of chloramine-T to a solution of sodium chloride is allowed to diffuse into a separate chamber that contains the solution mixture of protein and radioiodine[254,260]. The chlorine gas thus produced oxidizes the radioiodine and protein labeling occurs in about ten minutes[254]. The reaction is terminated by the addition of sodium metabisulfite[254,260]. This method minimizes the exposure of the protein to harmful reagents and satisfactory labeling of proteins has been reported[254].

Iodogen or Chloramide (1,3,4,6-Tetrachloro-3α,6α,diphenylglycouril)

Films of iodogen conveniently plated in reaction vessels from chloroform or methylene chloride solution have been found to react rapidly in the solid phase with an aqueous mixture of iodine and protein to yield iodinated proteins[267,268] with labeling efficiencies reported in the range of 70% to 80%[265]. No further reduction is needed to stop the reaction and the free radioiodide can be separated from the labeled protein by methods of gel filtration chromatography or dialysis[254,265]. Denaturation of the protein by this labeling method has been found to be minimal[267,268].

Electrolytic iodination

Oxidation by means of electrolysis at a constant current level has been employed to convert iodide to iodine in the place of chemical oxidants for the radioiodination of proteins[269]. The electrolytic cell has a platinum crucible as the anode which contains the protein solution and the radionucide and a platinum cathode surronded by dialysis membrane immersed in the protein-radioiodine solution[254,265]. A slow and controlled rate of electrolysis results in a steady liberation of radioiodine which labels the protein. The free unreacted radioiodide can then be removed from the labeled protein by conventional procedures.

The labeling procedure is mild and the proteins that have been radiolabeled with this technique have been found to retain high levels of immunologic and biologic activity[254]. The protein is not exposed to chemical oxidants and reductants, and labeling efficiencies of approximately 80% has been reported[254,260,265]. The labeling procedure can also be controlled by the amount of current or carrier iodide[254]. This technique, however, is complex to perform and specialized equipment is also required. Denaturing of unstable proteins have been found to occur due to prolonged exposure of protein to radioiodide, the dilution and the temperature of the electrolysis[254,265].

Enzymatic iodination

The enzyme Lactoperoxidase was first used by Marchalonis[270] to catalyze the oxidation of iodide in the presence of very small amounts of hydrogen peroxidase. Iodination is initiated by the addition of hydrogen peroxide to a mixture of protein, radioiodine and lactoperoxidase[254,260,265]. The reaction is stopped by the addition of cysteine or by dilution, and the free unreacted radioiodide can then be separated from the labeled protein by methods of gel filtration or dialysis[254,260]. A pH of 5.6 has been found optimal for this process of labeling[254,260]. Denaturation of the protein is minimal with this method of labeling as the protein is not being exposed to strong oxidizing or reducing agents and the biological and immunological properties of the original protein

has also been reported to be maintained[254,260,265]. Labeling efficiency has been found to be lower than some other methods but this method allows higher specific activity to be obtained as no carrier iodine is needed for the procedure[265]. Lactoperoxidase itself undergoes partial iodination, hence increasing iodine loss[254,265]. Glucose oxidase and lactoperoxidase have been used simultaneously to increase labeling efficiency[254].

Conjugation labeling methods

These methods were developed specifically to overcome the problems of direct radioiodination[254,260]. These methods offer the advantage of not having to expose the protein directly to oxidizing and reducing agents or to the radioiodine solutions[254,260]. It is possible to label different amino acids of the protein with these methods and hence, is especially useful when the protein lacks suitable tyrosine residues for direct iodination or when substitution of iodine directly into the tyrosyl residue of the protein destroys their activity[260]. These methods are more complex to perform and have a two stage reaction which usually results in lower iodination yields when compared to the direct methods[260].

N-Succimidyl hydroxyphenyl propionate (N-SHPP)

The N-hydroxysuccinimide ester of iodinated p-hydroxyphenyl propionic acid, first proposed by Rudinger and Ruegg[272] for protein labeling, has been used extensively for the radioiodination of various proteins by Bolton and Hunter[273]. N-SHPP is first radioiodinated by the chloramine-T method and extracted from the reaction mixture. This labeled ester is then used directly to react with primary amino groups of proteins to convert them to acyl derivatives at a pH of 8.4. The reaction is terminated by the addition of excess amino acid[260,265]. Protein denaturation due to oxidizing or reducing agents is virtually eliminated in this procedure. This technique is time consuming and has been reported to have an overall labeling efficiency of 10% to 30%[254].

Radioiodosulfanilic acid conjugation

Labeled diazotized iodosulfanilic acid has been found to react actively and form stable covalent bonds with functional groups of various amino acid residues in whole protein molecules[254]. Neutralized labeled diazonium salt is coupled to the protein when they are mixed together for about 10 to 15 minutes in an ice bath[254]. The reaction is terminated by the removal of free radioiodide[254]. Labeling efficiency of 80% has been reported with this method. This method is a substitute to the N-SHPP (Bolton-Hunter) technique[273] when it is especially undesirable to label terminal amino groups of a protein molecule[254].

Protein damage from radioiodination procedures

For the procedure of radioimaging, a protein should be labeled to a high enough specific activity to allow for good detection and still retain its original biological and immunological characteristics. Protein damage due to radioiodination procedures occurs when the degree of radioiodine substitution is high[254] and results in the loss of biological and immunological activity of the labeled protein with consequent a rapid clearance of the protein from the circulation usually by the liver after intravenous administration[254,260,265]. Protein damage may occur from a) the alteration in the physiochemical properties by the introduction of radioiodine atoms into the protein molecules in which the labeled protein may undergo considerable structural changes resulting in the alteration of biological activity, b) storage and labeling, in that structural alterations may result from the high levels of radiation which cause ionization and excitation of the protein molecules directly and indirectly and c) chemical damage due to the radioiodide, reagents and impurities in radioiodide solutions, and also from the exposure of the protein molecule to reagents, oxidizing and reducing agents, used in direct radioiodination methods[254,265]. Radioiodine labeled protein molecules have been observed to form macroaggregates during labeling and storage hence leading to loss in immunoreactivity and increased absorption to test-tubes and other containers[254]. Aggregated protein molecules

have been shown to localize in the lungs and in the reticuloendothelial system of the liver, spleen and bone marrow[254]. It has been recommended that an average incorporation of a maximum of one radioiodine atom per protein molecule be attained as any more iodine incorporation may alter the biological and immunological activity of the protein[260].

III. Materials and Methods

A. Preparation of protein solutions

Peanut Agglutinin

Affinity purified peanut agglutinin (PNA) was obtained from ChemBioMed, University of Alberta, in a salt- and sugar- free lyophilized form. The PNA was reconstituted with 0.01M phosphate buffer saline (PBS) to give a concentration of 1mg/mL. The reconstituted protein solution was then passed through a 0.22μ Millex-GS millipore filter (Millipore Corp., Bedford, MA.) into a sterile Falcon® tube (CanLab, Canada). The PNA solution was always used freshly prepared.

Anti-epiglycanin, murine IgM monoclonal antibody

Anti-epiglycanin is a murine IgM monoclonal antibody found to have high binding affinity to epiglycanin, a high molecular weight glycoprotein (500 000 daltons) found on the surface of the TA3/Ha murine mammary adenocarcinoma cells. Affinity purified anti-epiglycanin was kindly provided by Dr. B.M. Longenecker, Department of Immunology, University of Alberta, as a sterile solution. The protein solution was stored at 4° C and used within two weeks after obtaining it.

Bovine serum albumin

Powdered bovine serum albumin (BSA), prepared from pasteurized bovine serum albumin and low temperature solvent precipitation, was obtained from Sigma Chemical Company, St, Louis, Mo. The BSA was reconstituted with PBS to a concentration of 1mg/mL and passed through a 0.22μ millipore filter into a sterile Falcon® tube. The protein solution was used freshly prepared.

Nonspecific Rabbit IgG F(ab')$_2$ Fragments

Chromatographically purified nonspecific rabbit IgG F(ab')$_2$ fragments (Cappell Lab., Cochranville, PA.) was obtained in a lyophilized form with 0.1% azide preservative. The [redacted] was reconstituted with 0.01M PBS to a concentration of 1 mg/mL. The reconstituted protein solution was pla[redacted]por® dialysis membrane tubing (Spectrum Medical Industries Inc., Los Angeles.) and dialyzed overnight at 4° C against 0.01M PBS (1 litre) to remove the azide preservative. The dialyzed protein solution was then millipore filtered into a sterile Falcon® tube and used freshly prepared.

Nonspecific mouse IgM

Chromatographically purified mouse myeloma IgM (Miles Scientific, IL.) was obtained without any preservatives in the concentration of 1 mg/mL in 0.02M Tris buffered saline. The IgM was a combination of equal amounts of myeloma IgM isolated from ascites fluid generated by the tumor line TEPC 183 and MOPC 104E, both lines originating in BALB/c mice. The protein solution was stored at -80° C and used within two months after opening the sterile package.

49H.24, murine IgM monoclonal antibody

49H.24 is an IgM monoclonal antibody that was produced after the immunization of BALB/c mice with neuraminidase treated human red blood cells and was found to have high affinity for the synthetic Thomsen-Friedenreich (T) hapten βGal(1→3)αGalNAc but no reactivity for the TA3/Ha tumor cells[274] *in vitro*. The 49H.24 monoclonal antibody was kindly provided by Dr. B.M. Longenecker, Department of Immunology, University of Alberta, in an affinity purified solution.

B. Selection of organ specific TA3/Ha metastatic variants

The selection of organ specific TA3/Ha metastatic tumor variants was performed in the laboratory of Dr. B.M. Longenecker, Department of Immunology, University of Alberta according to the method described by Fidler[243] using the *in vivo* and *in vitro* selection system. Groups of strain A/J mice were injected intravenously via the caudal vein with 10^4 viable TA3/Ha tumor cells. These animals were then sacrificed between 14 and 18 days post-injection of the cells and the various organs examined for gross tumor nodules. Any individual lung and liver metastatic foci were dissected out, passed through a fine mesh stainless steel screen and cultivated *in vitro* (RPMI 1640 medium (Gibco) + 10% fetal calf serum + 5 mM 2-mercapto ethanol + gentamycin 50 μg/mL) for about a week. Cells obtained from different organ tumor lesions were cultured in different flasks. After the 1 week *in vitro* growth, 10^4 viable cells from each organ tumor growth culture were injected intravenously into A/J mice. This whole selection procedure was repeated for up to 8 times.

C. Quantitative protein analysis

The protein concentration of freshly reconstituted protein solutions were determined with the use of the Bio-Rad Protein Assay Kit (Bio-Rad Lab., Miss., Ont.) which is a dye binding assay based on the differential color change of a dye in response to various concentrations of protein[275]. A standard curve of 1 μg/mL to 1 000 μg/mL protein concentration versus absorbance was generated using the supplied bovine gamma globulin as the standard protein and Coomassie Brilliant Blue G-250 as the dye. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to protein occurs[276]. The absorbance of the solutions were read at 595nm on a Unicam SP1800 UV Spectrophotometer (CanLab, Canada) with a blank reference made up of 0.01M PBS in place of the protein.

A standard curve using PNA was compared to that using the supplied bovine gamma globulin standard and both the absorbance curves generated were found to be very similar. This

was also the case when using the non-specific mouse IgM protein as the standard for IgM protein quantitations. Hence, all further protein quantitations were performed using the supplied bovine gamma globulin as the standard protein. A standard curve was always generated each time a protein quantitation was needed.

D. Radioiodination of proteins

Quality control of radioiodine solutions

The radionuclidic purity of the radioiodine solutions, $Na^{125}I$ and $Na^{131}I$, (Iodination grade, Atomic Energy Canada) was determined by obtaining the gamma spectra of each solution in a calibrated multichannel analyzer (Canberra, Series 40 MCA) and comparing the spectra obtained to that of published spectras[254].

The radiochemical purity of each the radioiodine solutions was determined with the use of instant thin layer chromatography. Gelman® (Gelman, Ann Arbour, Michigan) silica gel instant thin layer 20 cm strips were spotted with approximately 20 kBq of each solution, air dried and allowed to develop for about 15 cm in 85% methanol:water before being cut into 1 cm strips and counted in a Beckman 8000 gamma counter for the areas of radioactivity.

Radioiodination procedure

2 μg (1 mg in 5 mL chloroform) of iodogen (1,3,4,6-tetrachloro3α6αdiphenylglycouril, Pierce Chemicals, Rockford, IL.) was coated onto the bottom of a 12x75 mm culture tube, using a gentle stream of nitrogen to evaporate the chloroform. 50 μg (1 mg/mL in 0.01M PBS), of PNA was added to the required amount of iodination grade $Na^{125}I$ (4 000 MBq/mL in 0.1N NaOH, Atomic Energy Canada) which had been neutralized with 50 μL 0.5M PBS. Routinely, 12 MBq was used for radioiodination of doses for biodistribution studies and 70 MBq for radioimaging studies. This mixture was transferred to the iodogen coated culture tube and incubated for 45 minutes at 23°C with occasional gentle agitation. The reaction mixture was

then transferred to another empty culture tube, 20 μL of 1M NaI was added and this mixture was incubated for 20 minutes at 23° C. The free unreacted iodide was separated from the labeled protein by gel filtration chromatography on a Biogel (Bio-Rad Lab., Mississauga, Ont.) P6-DG column pre-equilibrated with PBS.

In biodistribution studies where PNA was used simultaneously with either BSA or the nonspecific rabbit IgG $F(ab')_2$ fragments, 50 μg of each protein used was radioiodinated. PNA was always labeled with I-125 and the other protein was labeled with I-131 using the iodogen method of labeling. In biodistribution studies with anti-epiglycanin and 49H.24 or the nonspecific mouse myeloma IgM, anti-epiglycanin was always labeled with I-125 and the other proteins with I-131 using the same method of labeling as for PNA.

Separation of unreacted radioiodide

Biogel P6DG is a spherical desalting polyacrylamide gel with a molecular weight exclusion limit of approximately 6 000 Daltons and has a hydrated particle size range of 90-180 μm. The gel was prepared by allowing the supplied dry gel to hydrate overnight in 0.01M PBS, in a volume which was twice as much as the expected packed bed volume (1 gram of dry gel will form about 8 mL of packed volume). About 15 mL of the packed bed volume of the hydrated gel was poured into a 1.0x30cm Econo Column (Bio-Rad Lab., Mississauga, Ont.) and this was found to give a good separation and resolution of free unreacted iodide from the labeled protein when eluted with 0.01M PBS.

The elution profiles of unlabeled and labeled PNA and the other proteins used were determined using the prepared column by monitoring the eluate for levels of both protein and radioactivity. A flow through ultraviolet photometer (LDC Duo Monitor) set at 280 nm was used to detect protein absorbance in comparison to a PBS reference in a paired UV cell, and the eluate was also allowed to pass across the face of a shielded 3"x3" NaI(Tl) crystal detector attached to a single channel analyzer. The elution profiles were recorded on a dual pen Fisher Recordall 5000 recorder, with both the radioactivity and optical density profiles of the protein

and free unreacted iodide recorded.

0.5 mL of 1% BSA in PBS was passed through the column and the column washed with about 10 mL of PBS before the application of the radioiodination mixture. The mixture was applied onto the column bed and allowed to drain in before washing the mixture into the bed with PBS. The column was then eluted with PBS. Just before the pre-determined void volume of the column was fully collected, 18 eight drop fractions were collected into Eppendorf® tubes, and after the last fraction was collected, the column was washed extensively with PBS.

The fractions collected from the separation procedure were measured for radioactivity in a Picker® Isotope Dose Calibrator and the fractions with the bulk of the radioactivity were pooled, mixed well and used. The free unreacted iodide has been determined to elute off the column after all the 18 fractions have been collected.

Quality control of the labeled proteins

Trichloroacetic acid (TCA) precipitation

Small aliquots of about 2 μL to 5 μL of the pooled fractions were diluted to 0.6 mL with 1% BSA in PBS in an Eppendorf® tube and counted for radioactivity. Then, 0.4 mL of 25% TCA was added to the mixture and the denatured protein was mixed well on a vortex mixer, centrifuged in an Eppendorf® centrifuge for 5 minutes, washed twice with PBS and the pellet, as well as the combined supernatant washings were recounted for radioactivity.

Instant thin layer chromatography (ITLC)

Small aliquots of the purified radioiodinated protein were spotted onto 20 cm Gelman® ITLC strips, air dried and allowed to develop to about 15 cm in 85% methanol in water solvent system. The strips were then cut into 1 cm segments which were counted for radioactivity.

E. Peanut agglutinin (PNA) studies

In vitro studies

Radiochemical stability of iodinated PNA

The rate of deiodination of radioiodinated PNA during storage at 4° C was determined by methods of TCA precipitation and ITLC analysis. A small aliquot of the radioiodinated PNA solution was diluted to 5 mL with PBS and stored at 4° C. Samples were then taken from the dilution preparation and used for the study.

Trichloroacetic acid precipitation

On days 0, 1, 2, 3, 5, 7, 14 and 30 after radioiodination of PNA, 100 μL of the diluted PNA solution was mixed well with 500 μL of 1% BSA in PBS in an Eppendorf® tube. 400 μL of 25% TCA was added to the mixture and the denatured protein was mixed well on a vortex mixer and counted for radioactivity. The denatured protein was then centrifuged in an Eppendorf® centrifuge for 5 minutes, washed twice with 0.01M PBS and recounted for radioactivity.

Instant thin-layer chromatography

On days 0, 1, 2, 3, 5, 7, 14 and 30 after radioiodinatiion, 20 μL of the diluted PNA solution was spotted onto 20 cm Gelman® silica gel ITLC strips, air dried and allowed to develop to about 15 cm in 85% methanol in water solvent system. The developed strips were then cut into 1 cm segments and counted for radioactivity.

Biological activity of radioiodinated peanut agglutinin

Studies with neuraminidase treated human red blood cells

Neuraminidase treatment

Blood from group types A, B, AB and O, freshly collected from normal healthy donors, were kindly donated by the Canadian Red Cross Society Blood Transfusion Service. The T antigen in the human red blood cells (RBC) was exposed by neuraminidase treatment. Two millilitres of whole blood was washed twice with 10 mL of 5% fetal calf serum (FCS) in PBS. 100 μL of packed RBC from each of the blood group types was incubated with 1 mL *Clostridium perfringens* neuraminidase (Sigma Chemical Co.) at a concentration of 1 unit/mL and 2 mL PBS for 45 minutes at 37° C. Control RBC were treated with normal saline. After treatment with neuraminidase, the RBCs were washed four times with 10 mL volumes of 5% FCS in PBS and resuspended in PBS to a final concentration of 0.3% (v/v).

In vitro binding study

Radioiodinated PNA (0.5 μg) was placed in Eppendorf® tubes and counted for radioactivity. To the counted tubes, 0.5 mL of 0.3% N'RBC or control RBC was added and the mixture incubated for 30 minutes on ice with occasional agitation. The mixture was then centrifuged for 5 minutes at 16 000 x G in an Eppendorf® centrifuge, washed three times with PBS, and the pellet counted for radioactivity. Galactose (0.1 M) was added to some of the tubes prior to incubation to determine the specificity of binding.

Binding to tumor cells and asialo GM-1 synsorb

Various tumor cell lines were incubated with ^{125}I-PNA to determine the binding activity of this protein to the tumor cells. The RI lymphoma tumor cell line which has been demonstrated to bind PNA *in vivo* and *in vitro*[45,46], the murine TA3/Ha mammary adenocarcinoma tumor cells, the selected lung and liver specific metastatic variant of

the TA3/Ha mammary adenocarcinoma cell line, the TA3/St tumor cells and the non-T antigen expressing EL4 tumor cell line[46] were used in this study. The various cells used were maintained in culture in RPMI 1640 medium (Gibco) supplemented with heat denatured fetal calf serum at a concentration of 10% and 2 mM L-glutamine. The cells were harvested in the log phase of growth and greater than 95% of the cells excluded trypan blue. One million cells from the tumor lines RI lymphoma, TA3/Ha mammary adenocarcinoma, TA3/St and EL4 were incubated with approximately 1.8 μg PNA on ice for 30 minutes with occasional agitation while 10^5 cells from the selected lung and liver specific metastatic variants of the TA3/Ha mammary adenocarcinoma cell line were incubated with approximately 0.2 μg PNA under the same conditions. Following the period of incubation, the cells were centrifuged for 5 minutes in the Eppendorf® centrifuge, washed twice with 1 mL 0.01M PBS and the pellet counted for radioactivity.

Ten milligrams of asialo GM-1 sorb, a synthetic immunoadsorbent (Chembiomed Ltd.) containing the immunodominant structure βDgalactosyl (1→3)β N-acetyl D-galactosamine, was incubated with 0.02 μg ^{125}I-PNA in 1 mL PBS and tumbled overnight followed by centrifugation, two washings with 1 mL PBS and then was counted for radioactivity. The specificity of the binding was determined by the inhibition of binding in the presence of galactose.

In vivo studies

Animal tumor models

Six to eight week old male A/J mice weighing about 20 g were used for biodistribution and imaging studies. Solid tumor bearing mice used for studies had been inoculated in the right flank with 10^6 viable TA3/Ha tumor cells. The tumors were allowed to grow in the mice for 7-10 days resulting in well vascularized, well defined solid tumors. In the lung and liver metastatic tumor studies, the mice were inoculated intravenously via the caudal vein with 10^6 viable TA3/Ha tumor cells of either the

specific lung or liver variant selection. Control mice in these studies were treated with normal saline. Mice bearing the TA3/Ha specific lung variant selection tumor cells were used 10 days post-inoculation of cells and groups of mice bearing the liver variant selection tumor cells were used after 12 days and 18 days following the intravenous inoculation of the tumor cells for the detection of macrometastasis. Mice that were injected intraperitonealy with 10^6 TA3/Ha cells were used 7 days post-injection of cells.

All the mice used in the various studies were maintained on standard laboratory food (Wayne® Lab-Blox, Chicago, IL.) and ordinary tap water. However, three days before any study, the mice were put on 0.01% KI drinking water.

Biodistribution studies

Intravenous injections of the radiopharmaceutical were made using Monoject® insulin syringes to minimize the amount of residual radiotracer in the needle and syringe. The total volume of radioiodinated protein/s injected per mouse was 0.2 mL. In single label experiments, I-125 was used as the label, and in dual label experiments, I-131 was used as the other label. All the syringes used were counted for radioactivity in a whole body counter before and after injection to determine the relative amount of radioactivity injected into the animal. Three extra doses were drawn up in syringes and the contents of each syringe were diluted fifty fold. From these dilutions, 0.2 mL aliquots were used as injection standards to correlate the radioactivity determined in the excised tissue samples to the injected dose.

The mice were sacrificed by cardiac puncture exsanguination under light ether anesthesia. Various tissues of interest and the remaining carcass were collected and weighed in tared Fisher® counting vials. The weighed samples were then counted for radioactivity along with the injection standards in a programmable automated NaI gamma well counter, Beckman 8000 Gamma Counter,. In single label studies with I-125, the coincidence method of counting was used as the absolute determination of I-125 radioactivity. In dual label studies, the I-125 radioactivity was corrected for the

spillover counts occuring in the I-125 channel due to I-131 counts. The amount of radioactivity injected per animal was about 150 kBq for each protein for biodistribution studies and about 2 MBq for ^{125}I-PNA for imaging studies.

Whole body retention of peanut agglutinin in TA3/Ha tumor bearing mice

Groups of mice were injected with 10^6 viable TA3/Ha tumor cells either subcutaneously or intraperitonealy. Control mice were treated with normal saline. Seven days post-inoculation of the cells, the mice were injected with ^{125}I PNA intravenously (1.0 μg PNA; 150 kBq) and each mouse was then housed separately. At times 0, 3, 8, 24 and 48 hour post-injection of ^{125}I PNA, the mice were counted for radioactivity in a whole body counter.

Dosage determination of the peanut lectin

Groups of male A/J mice bearing a solid TA3/Ha tumor were injected with either ^{125}I-PNA (0.1 μg) and ^{131}I-BSA (0.1 μg), ^{125}I-PNA (1.0 μg) and ^{131}I-BSA (1.0 μg), ^{125}I-PNA (10.0 μg) and ^{131}I-BSA (10.0 μg), or 60.0 μg ^{125}I-PNA alone. Twenty four hours post-injection of the radioiodinated proteins, the mice were sacrificed and the tissues of interest excised and counted for radioactivity.

Biodistribution of I-125 peanut lectin at various time intervals

Mice bearing the TA3/Ha solid tumor in the right flank were injected intravenously with ^{125}I-PNA (1.0 μg) and nonspecific rabbit IgG ^{131}I-F(ab'), (1.0 μg). Groups of 8 to 10 mice were sacrificed at 3, 6, 24, 48 and 72 hours post-injection of the radioiodinated proteins and the tissues of interest were removed and counted for radioactivity.

Biodistribution of I-125 peanut lectin in mice bearing the selective TA3/Ha lung, specific metastatic tumor variant

Male A/J mice were inoculated intravenously with 10^6 TA3/Ha tumor cells of the specific lung variant selection, Lung VIII. Control mice were treated with normal saline. Ten days post-inoculation of the cells, the mice were injected intravenously with ^{125}I-PNA. Eight hours post-injection of the protein, the mice were sacrificed and the tissues of interest were removed and counted for radioactivity.

Biodistribution of I-125 peanut lectin in mice bearing the selective TA3/Ha liver specific metastatic tumor variant

10^6 viable TA3/Ha tumor cells of the specific liver variant selection, Liver V, were injected intravenously via the tail vein into male A/J mice. Controls were treated with saline. Twelve days post-injection of the tumor cells, a group of ten tumor bearing mice and ten control mice were injected intravenously with ^{125}I-PNA (1 μg PNA; 150 kBq). Twenty four hours post injection of the radioiodinated protein, the mice were sacrificed and the tissues of interest excised and counted for radioactivity. Another group of ten tumor bearing mice and ten control mice were injected with the radioiodinated PNA eighteen days post injection of the tumor cells. These mice were sacrificed twenty four hours post injection of the radioiodinated PNA and the tissues of interest were removed and counted.

Whole body gamma scintigraphic imaging

Male A/J mice bearing solid TA3/Ha tumors in the right flank were injected with 2 MBq (4 μg) ^{125}I-PNA and serial scintigraphic images of the mice, lightly anesthesized with 1.3 mg ketamine and 0.13 mg xylazine intraperitonealy 10 minutes prior to imaging, were collected on a Searle PhO/Gamma IV camera (50000 counts, pinhole collimator, 5 mm aperture) and stored on an ADAC CAM II clinical acquisition processing unit. Each mouse was held down to a prone position on a positioning board by means of a masking tape and images were taken of the posterior view at time periods of 6, 24, 48 and 72 hours post-injection of ^{125}I-PNA. The same

procedure was repeated for mice bearing either the specific lung or liver variant selection of the TA3/Ha tumors, but the images were taken at 8 hours and 24 hours post-injection of ^{125}I-PNA for the specific lung variant selection and the specific liver variant selection respectively.

Autoradiography

Lung and liver samples found with metatastatic infiltrations in the mice bearing either the TA3/Ha Liver or Lung specific variant selection were excised at 24 hours and 5 days post-injection of ^{125}I-PNA (4 μg; 2 MBq). The tissue samples obtained were embedded in embedding medium (Tissue Tek II, Miles Laboratories, Naperville, IL.) at -25° C and sections of the tissues of 4 μ to 6 μ thick were cut out with a cryostat microtome and placed onto microscopic slides. Alternate sections were stained with hematoxylin and eosin while sections intended for autoradiography were air dried and placed in apposition to ultrofilm ^{3}H film (LKB, Rockville, MD.) under darkroom conditions. The autoradiograms were exposed in dark cassettes for 5 to 7 days, then developed for 4 minutes in 10% dilution of Kodak D-19 developer, fixed for 8 minutes in a 10% dilution of Kodak fixer and rinsed thoroughly in distilled water.

FITC-PNA Fluorescence Studies

Fixed cyotsat sections were serially rehydrated by 2 minute washes in xylene; 100%, 95%, 80% and 70% ethanol series, followed by 2 minute washes in PBS, pH 7.4. The rehydrated sections were washed once with 10% FCS in PBS, then covered with FITC-PNA (100 μg/mL, E.Y. Laboratories, San Mateo, Ca.) in PBS containing 10% FCS and incubated for 20 minutes. The slide were then placed in slide dishes, washed with two changes of PBS while being gently stirred for 20 minutes at 4° C. The sections were then mounted with glycerol media (50% glycerol in PBS) and examined with a Nikon Optiphat epifluorescent microscope.

Biodistribution of $Na^{125}I$ in TA3/Ha tumor bearing mice

Mice bearing solid TA3/Ha tumors were injected intravenously with 150 kBq $Na^{125}I$ and sacrificed for biodistribution studies at 6, 24 and 48 hours post-injection of the radioiodide. The mice had been on 0.01% KI drinking water 3 days prior to and throughout the study. The tissues excised were the same as the ones taken from all the other biodistribution studies and were examined for uptake of radioiodine.

F. Anti-epiglycanin studies

In-vitro studies

Binding to N'RBC and TA3/Ha tumor cells

0.5 μg of ^{125}I Anti-Epiglycanin was placed into Eppendorf® tubes and counted for radioactivity. To the counted tubes, 0.5 mL of a suspension of 0.3% neuraminidase treated human red blood cells (N'RBC) or control red blood cells was added and incubated on ice with occasional agitation for 30 minutes. The incubation mixture was centrifuged, washed twice with PBS, and the resulting pellet counted for radioactivity.

Viable TA3/Ha cells (5×10^6) were incubated in the pre-counted tubes containing ^{125}I anti-epiglycanin for 30 minutes on ice with occasional agitation. The mixture was centrifuged, washed twice with PBS and the pellet counted for radioactivity.

The specificity of the binding of ^{125}I anti-epiglycanin to N'RBC and TA3/Ha cells was determined by the presence of galactose in the incubation mixture.

Effect of anti-epiglycanin binding to N'RBC in the presence of peanut lectin.

^{131}I-PNA (0.25 μg) was mixed with ^{125}I anti-epiglycanin in Eppendorf® tubes and counted for radioactivity in the Beckman 8000 gamma counter using a dual label counting program designed to correct for spillover of ^{131}I counts occurring in the ^{125}I

window. To these pre-counted tubes, 0.5 mL of either 0.3% N'RBC or control RBC was added and the tubes were incubated on ice for 30 minutes with occasional agitation. The incubation mixture was centrifuged for 5 minutes, washed twice with 1 mL PBS and the resulting pellet was counted for radioactivity using the dual label program.

Effect of anti-epiglycanin binding to TA3/Ha tumor cells in the presence of the peanut lectin

^{125}I anti-epiglycanin (0.5 μg) was put into Eppendorf® tubes and the radioactivity content was determined. To these pre-counted tubes, 0.0 μg, 0.5 μg, 50 μg, 120 μg, 250 μg and 500 μg of PNA was added and mixed well. Viable TA3/Ha tumor cells which excluded more than 95% trypan blue and grown in culture for two weeks were added to the protein mixture and incubated on ice for 30 minutes with occasional agitation. The mixture was centrifuged for 5 minutes, washed twice with 1 mL PBS and then counted for radioactivity.

Effect of peanut lectin binding to TA3/Ha tumor cells in the presence of anti-epiglycanin

0.5 μg of ^{131}I-PNA was counted for radioactivity and mixed with 0.0 μg, 6.0 μg, 12.5 μg, 25 μg and 50 μg anti-epiglycanin in Eppendorf® tubes. Viable TA3/Ha tumor cells were incubated with the protein mixture for 30 minutes on ice with occasional agitation. The mixture was centrifuged for 5 minutes, washed twice with PBS and the pellet counted for radioactivity.

In vivo studies

Biodistribution of ^{125}I anti-epiglycanin monoclonal IgM antibody in TA3/Ha tumor bearing mice

Male A/J mice were inoculated subcutaneously with 10^6 viable TA3/Ha tumor cells in the right flank. The tumors were allowed to grow for 10 days producing solid,

well defined palpable tumors. The mice were then injected intravenously with ^{125}I anti-epiglycanin (1.0 μg) and ^{131}I 49H.24 (1.0 μg). The monoclonal IgM antibody, 49H.24, was used here as the nonspecific IgM antibody localizing control. Both the radioiodinated IgM monoclonal antibody solutions were subjected to ultracentrifugation at 100 000 x G in a Beckman Air-fuge for 24 hours and only the top layers of the centrifuged solutions were used. The mice were put on 0.01% KI drinking water 3 days prior to and throughout the study. Groups of 8 mice were sacrificed at 6, 24, 48 and 72 hours post-injection of the radiotracers and the tissues of interest were excised and counted for radioactivity in the Beckman 8000 gamma counter using the dual label program.

24 hour biodistribution of anti-epiglycanin in TA3/Ha tumor bearing mice

Mice bearing solid TA3/Ha tumors in the right flank were injected intravenously with ^{125}I anti-epiglycanin (1.0 μg) and ^{131}I non-specific mouse IgM (1.0 μg). These proteins radioiodinated proteins had been centrifuged for 24 hours x 100 000 G in a Beckman Air-fuge and only the top layer was used for injection. 24 hours post injection of the radioiodinated proteins, the mice were sacrificed and the tissues of interest excised and counted for radioactivity using the dual label program.

Biodistribution of anti-epiglycanin and the peanut lectin in TA3/Ha tumor bearing mice

Groups of mice bearing the TA3/Ha solid tumor were injected intravenuosly with either ^{125}I anti-epiglycanin (1.0 μg), ^{131}I PNA (1.0 μg) or a combination of ^{125}I anti-epiglycanin (1.0 μg) and ^{131}I PNA (1.0 μg). The ^{125}I anti-epiglycanin used was obtained from the top layer of the protein solution that had been centrifuged for 24 hours at 100 000 x G in the Beckman Air-fuge. The mice were sacrificed 24 hours post-injection of the radioiodinated proteins by cardiac puncture exsanguination under light ether anesthesia and the tissues of interest were removed and counted for

radioactivity.

Whole body gamma scintigraphic imaging

Male strain A/J mice were inoculated subcutaneously in the right flank with 10^6 viable T-1.5 Ha tumor cells and the tumor was allowed to grow for about 6 days producing a solid, well-defined and palpable tumor of about 150 mg in size. Three days before the study was started, these mice were put on 0.01% KI drinking water. These mice were then injected with 2 MBq I-125 labeled anti-epiglycanin (4 μg) intravenously and at 24, 48 and 72 hours post-injection of the radiotracer, each mouse was lightly anesthesized (1.3 mg. ketamine and 0.13 mg. xylazine intraperitoneally) and held down to a prone position onto a board by means of masking tape. Scintigraphic images were then taken from the posterior view using a Searle Pho/Gamma IV camera with a pinhole collimator. The data was acquired onto a floppy disk with the aid of the ADAC CAM II clinical acquisition processing unit.

IV. Results and discussion

A. TA3/Ha animal tumor model

The investigation of the potential utility of peanut (*Arachis hypogaea*) lectin (PNA) as a diagnostic radiopharmaceutical for the radioimmunodetection of tumors requires an appropriate animal tumor model. The animal tumor model selected should preferably resemble, as close as possible, a clinical situation in which PNA is investigated for. The peanut lectin has been reported to have high binding affinity for glycoproteins containing the terminal sequence βDgalactosyl(1→3)α-D-GalNAc[30,17,9] which is the immunodeterminant carbohydrate structure of the Thomsen-Friedenreich (T) antigen[27]. The T-antigen which has been reported to be a tumor associated antigen in man[50] has been found to be present in a number of normal glycoproteins in a cryptic form masked by N-acetylneuraminic acid but in an unsubstituted form on several carcinomata especially in the breast[54,55,58], lung[53] and gastrointestinal tract[54,55,57]. T-antigen has also been postulated as having a possible role in the process of cancer invasion and metastasis[36]. *In vitro* binding studies have been well documented on PNA interaction with T-antigen type receptors on various human tumor sites such as human mammary carcinoma[50,54,58,59] and carcinomas of the gastrointestinal tract[134,135] and animal tumor cell lines such as the RI lymphoma, TA3/St mammary carcinoma and the TA3/Ha mammary carcinoma[45].

The TA3/Ha spontaneous murine mammary adenocarcinoma cells have been demonstrated to bind the peanut lectin avidly[45]. This tumor model resembles human adenocarcinoma in its spontaneous origin and its content of human blood group N-like antigen specific structures[39]. Furthermore, intact TA3/Ha tumor cells have also been reported to absorb the anti-N lectin *Vicia graminea* and human and animal anti-T antibodies[50]. The TA3/Ha tumor cells shed a high molecular weight (500 000 daltons) glycoprotein molecule, epiglycanin, into the serum and ascites fluid during the *in vivo* growth in animals[232,237,240]. The shed epiglycanin molecule expresses the immunodeterminant structure βDgalactosyl(1→3)DGalNAc

of the Thomsen-Friedenreich antigen[43,44] and 60% of the oligosaccharides found on the surface of the epiglycanin molecule have been found to be T-antigenic in structure[246]. Epiglycanin has also been found to possess immunodeterminant structures for the MN antigens as well as receptor sites for several other lectins[43,234,241]. The TA3/Ha tumor cells have been found to be allotransplantable as well as xenotransplantable in the various animals studied, and this phenomena which has been extensively documented has been ascribed largely to the presence of the TA3/Ha tumor cell surface epiglycanin[232,237,240].

Metastatic variants of the TA3/Ha tumor which selectively grow in the liver or lungs upon the intravenous injection of the tumor cells into strain A mice have been isolated and were obtained from Dr. B.M. Longenecker, Department of Immunology, University of Alberta. Epiglycanin was found to be present on the tumor cell surface of these metastatic TA3/Ha variants and a correlation has been observed to exist between the metastatic potential of the TA3/Ha tumor cells and its epiglycanin content[236]. Selected TA3/Ha metastatic lung and liver variants have also been found to maintain a high expression of the Thomsen-Friedenreich antigen as well[45]. The TA3/Ha animal tumor model is thus used for the study of radioiodinated peanut lectin as a potential tumor radiolocalizing agent as this tumor line bears some similarity to human adenocarcinomas[39]. This animal tumor model with its availability of selected metastatic variants also allows the study of radiolabeled peanut lectin as a potential imaging and detection agent of organ specific metastases with scintigraphic procedures. Furthermore, the TA3/Ha tumor which sheds epiglycanin into the serum[232,237,240] would be a good model to determine whether shed tumor antigens which bind readily to the radiolocalizing agent would cause any interference in the radioimmunodetection of the tumor with radioiodinated peanut lectin and monoclonal antibodies.

B. Peanut Lectin

Radioiodination of proteins

The radioiodine solutions, $Na^{123}I$ and $Na^{131}I$ (Iodination grade, Atomic Energy, Canada) were obtained from the Edmonton Radiopharmaceutical Centre and checked for radionuclidic and radiochemical purity. The radionuclidic purity was determined using a calibrated multichannel analyzer (MCA series 40, Canberra) with a sodium iodide crystal detector. Using a small aliquot of the radioiodine solutions, the gamma spectra obtained from the multichannel analyzer for each of the radionuclides was compared to that of published spectra in the literature[235] and found to be similar. Radiochemical purity of the radioiodine solutions were performed by applying approximately 20 kBq spots onto silica coated instant thin layer chromatography paper strips, air dried and developed by the method of ascending chromatography in 85% methanol:water. It was found that almost all of the radioactivity (>97%) was concentrated at the solvent front. Sodium iodide migrates with the solvent front with an Rf value of 1.0 while other oxidized forms of iodine such as sodium iodate remain at the origin with the 85% methanol:water solvent system. On the basis of these two quality control tests, it was assumed that the radioiodine solutions used were pure both in a radionuclidic and radiochemical form. The proteins used in the studies, peanut lectin and the non-specific control proteins such as bovine serum albumin(BSA) and F(ab')$_2$ IgG fragments, were reconstituted from their various supplied form with 0.01 molar phosphate buffer-saline to a final concentration of 1 mg/mL. Radioiodination of these proteins were carried out using the iodogen method[267,268]. Iodogen (1,3,4,6-Tetrachloro-3α,6α,diphenylglycouril), a mild oxidizing agent which is stable and insoluble in water can be plated out onto reaction vessels to permit a solid phase reaction with aqueous solutions of I^- and proteins[267]. This simple method of iodination has been found to result in minimal damage to the proteins but can give higher iodine labeling efficiencies compared to the more common methods of chloramine-T and lactoperoxidase[267,268]. The need of a reducing agent to terminate the labeling process in this

method is also eliminated thereby further reducing the exposure of the protein to potentially denaturing and damaging agents. Various proteins that have been labeled with the iodogen method to high specific activities with iodine-125 has been found to be stable for up to 3 months[265]. The various proteins used in the studies here were routinely radioiodinated using the iodogen method resulting in labeling efficiencies of at least 50% to give specific activities of approximately 150 kBq/μg (approximately 0.2 atoms of iodine per molecule) for animal biodistribution studies and about 740 kBq/μg (approximately 1 iodine atom per molecule) for radioimaging studies using 2 μg of iodogen for every 50 μg of protein to be iodinated. Separation of free unreacted iodide from the labeled protein was achieved with good separation resolution using about 15 mL of a packed bed volume of a desalting gel, Biogel P6DG (Bio-Rad Lab., Miss., Ont.), which has a molecular weight exclusion limit of approximately 6 000 daltons. Using phosphate buffer saline as the eluting solution in this column, it was estimated that free unreacted iodide was eluted off the column about 5 mL after the labeled protein, thus resulting in a good separation of the protein. The percentage of the radioactivity in the final product as labeled protein was routinely found to be greater than 98% by the method of trichloroacetic acid precipitation with ice-cold 25% TCA.

Radiochemical stability

The rate of deiodination of labeled peanut lectin that was stored at 4° C was determined using the methods of TCA precipitation and instant thin layer chromatography with 85% methanol:water solvent system. Aliquot samples of the radioiodinated peanut lectin were taken at various time intervals after radioiodination and tested by both methods for up to 30 days. Each test was done in triplicates to reduce any possible errors. It was found that radioiodinated PNA stored at 4° C was still greater than 95% precipitable with 25% TCA seven days post-iodination and about 90% precipitable thirty days post-iodination. Using instant thin layer chromatography with 85% methanol:water as the solvent system, the results obtained complemented that of the TCA precipitation tests fairly well. In a 7 day old sample of

radioiodinated peanut lectin, it was found that there was still about 90% of the total radioactivity at the origin of the developed chromatography strip. Free iodide will migrate with the solvent front with a Rf value of about 1.0 while labeled PNA will remain close to the origin. Hence, it appears that the radiochemical purity of radioiodinated peanut lectin remains relatively stable for at least a week post-iodination when stored at 4° C at pH 7.4.

Cell-binding studies

The biological activity of peanut lectin after radioiodination was determined in order to assess whether any damage or changes in the immunological properties of PNA were caused by the radioiodination process or by the presence of radioactive iodine atoms on the protein structure. This can be readily achieved by a number of *in vitro* binding studies and the more common and simple test is to visually check the hemagglutination activity of PNA on neuraminidase treated red blood cells (N'RBC). A simple but yet quantitative method is to determine the percentage binding of a known amount of radiolabeled PNA to a known number of neuraminidase treated red blood cells. Table 1 gives the result of such a study. The blood from various group types A, B, AB and O were kindly donated by the Canadian Red Cross society blood transfusion service after the blood had been freshly collected and the T-antigen on the red blood cells was exposed by treating the cells with 1 unit of neuramindase. 0.5 μg of radioiodinated PNA was used in each of the determinations to 0.5 mL of a 0.3%(v/v) of neuramindase treated red blood cells. The untreated red blood cells of each blood group type served as controls. Each determination was performed in triplicate.

Peanut lectin is shown to have high avidity for the T-antigen present on the exposed red blood cells regardless of blood group type whereas there was an insignificant amount of PNA binding observed with the normal untreated red blood cells. Visible hemagglutination was observed upon incubation of the peanut lectin with the neuramindase treated red blood cells which was not seen with the normal red blood cells. The slight variation in binding observed with the various blood group types of neuraminidase treated red blood cells is most probably

Table 1: *In vitro* binding of radioiodinated PNA to human erythrocytes[1].

	Blood group type			
Human erythrocytes	A	B	AB	O
N'treated cells	26.00±1.27	24.80±1.79	31.00±3.00	28.80±1.59
+ 0.01M galactose	9.40±0.90	10.05±2.40	12.95±0.95	9.50±1.30
(approximately 0.75x10^7 cells)				
Control cells	2.20±0.05	3.50±0.75	3.40±0.10	3.30±0.65
+ 0.01M galactose	2.02±0.10	3.20±0.20	2.90±0.22	2.90±0.04
(approximately 1.0x10^7 cells)				

[1]Values are expressed as the percentage ± standard deviation of the added ^{125}I-PNA (20 kBq/0.5 μg) after incubation and 3 washes with PBS.

due to the difference in the total number of cells used in each blood group type. The binding of PNA to N'RBC was seen to decrease significantly in the presence of galactose hence, demonstrating the sugar specificty of the peanut lectin. The control red blood cells did not show any appreciable binding to the peanut lectin and the presence of galactose in the mixture caused little, if any, inhibition of PNA-RBC interactions therby implying that the observed (3.0%) binding of PNA to the red blood cells was probably not due to PNA-T antigen interaction. It could be possible that some of the radioactivity seen in the control red blood cells after washing was due to the entrapment of some PNA solution between the packed red blood cells.

Another test to determine the immunological binding activity of radioiodinated peanut lectin is to incubate the protein with the asialo GM-1 synsorb, a synthetic immunoadsorbent which bears the β configuration of the T-antigenic disaccharide. Upon incubation of the ^{125}I-PNA (0.02 μg) with the immunoadsorbent, it was found that 79.9 $\pm$ 1.5% of the added radioactivity remained bound to the synsorb after two washings with 1 mL PBS. This level of binding was seen to decrease to 13.9 $\pm$ 6.2% in the presence of 0.05 molar galactose. This binding assay and the one done with the N'RBC demonstrates the retention of the immunological binding activity of the peanut lectin after the radioiodination procedure.

Tumor cell binding

The avidity of the peanut lectin for various tumor cells *in vitro* was studied. Table 2 illustrates the results of such a study. The RI lymphoma tumor cell line has been shown to express PNA binding sites *in vitro* and also take up radiolabeled PNA in the RI lymphoma animal tumor model[45,46]. The TA3/Ha murine mammary adenocarcinoma tumor cells have been known to absorb human and animal anti-T antibodies[50] as well as PNA[45]. This same tumor line also sheds a high molecular weight glycoprotein, epiglycanin which expresses the immunodeterminant carbohydrate structure of the T antigen in 60% of its oligosaccharides chains[246]. The metastatic variants of the TA3/Ha tumor cell line have been selected to produce liver or lung metastatic tumors upon the intravenous injection of the cells into the animals and the

Table 2: Binding of radioiodinated peanut lectin to various murine tumor lines[1]

Tumor line	Percentage bound[2]
RI lymphoma[3]	1.35±0.20
TA3/St[3]	2.14±0.60
EL 4[3]	0.40±0.02
TA3/Ha[3]	7.53±0.40
+ 0.02M galactose[3]	1.10±0.10
+ 0.10M galactose[3]	completely inhibited
TA3/Ha liver variant[4]	18.89±2.39
TA3/Ha lung variant[4]	21.00±0.46

[1] all studies were performed in triplicates and the cells were washed twice with 0.01M PBS before counting.

[2] values presented as the mean ± standard deviation.

[3] percent binding of ^{125}I-PNA (3.7 kBq/1.8 μg) to 10^6 tumor cells after 1 hour incubation on ice.

[4] percent binding of ^{125}I-PNA (3.7 kBq/0.2 μg) to 10^5 tumor cells after 1 hour incubation on ice.

metastatic potential of the TA3/Ha tumor cells has been found to be dependent upon the tumor cells' surface epiglycanin content[236]. The non-epiglycanin secreting TA3/St subline is a non-allotranplantable tumor cell line that exhibits some PNA receptor sites. A non-T antigen expressing tumor cell line EL4 is used here as a control. The results in table 2 indicate that PNA has high binding avidity for the TA3/Ha tumor cells and that this binding affinity can be blocked by the presence of galactose. The selected lung and liver specific metastatic tumor variants of the TA3/Ha mammary adenocarcinoma also displayed active PNA binding and hence, it appears that the metastatic tumor variant cells of the TA3/Ha retained the T-antigenic specificity on their surface. This would imply that epiglycanin is still present on the surface of the metastatic tumor cells after the selection of these cells for its metastatic growth from the parent TA3/Ha solid tumor line. This then indicates that radioiodinated PNA may be potentially useful in the localization and external imaging detection of metastatic lesions of a primary tumor which PNA has high avidity for *in vivo*.

Tissue biodistribution studies

Preliminary findings

Preliminary biodistribution studies were performed on male, strain A/J, mice bearing subcutaneously inoculated TA3/Ha tumors in the right flank and TA3/St tumors in the left flank. Approximately 1 x 10^6 tumor cells of each line was injected into the respective sites in the mice and the tumor was allowed to grow for seven days resulting in well vascularized solid tumor masses of about 175 - 275 mg. A dual isotope label was performed where ^{125}I-PNA (1.0μg) and ^{131}I-nonspecific rabbit IgG F(ab')$_2$ fragments (1.0μg) were injected intravenously via the caudal vein into the mice seven days post-injection of the tumor cells. At time intervals of 24, 48 and 72 hours post-injection of the radiotracer, groups of three mice were sacrificed and the organs of interest were dissected out whole. Blood samples were drawn and the radioactivity in

the blood was extrapolated to the entire blood pool where entire blood pool was estimated to be 6.5% of the total body weight. The tissue samples and the excised tumor masses were counted for radioactivity along with the injection standards using the dual isotope counting program which accounts for spillover counts due to I-131 in the I-125 counting channel (Beckman 8000 gamma counter).

The results of this study are presented in table 3 as the percent uptake of the injected dose per gram of tissue. A high TA3/Ha tumor uptake of ^{125}I-PNA is seen in comparison to most of the other tissues including the TA3/St tumor. A rapid blood clearance of ^{125}I-PNA was evident (table 3). Blood concentration of the labeled PNA was taken as a nontarget tissue and the TA3/Ha tumor to blood ratio is given in figure 2 for the various time intervals. The high TA3/Ha tumor uptake of PNA combined with the rapid blood clearance of the radiotracer seen in this study makes radiolabeled PNA a potential radioimaging agent in this animal tumor model and hence, warrants further investigation of radiolabeled PNA as a tumor localizing agent. A more informative manner to determine the specificity of the tracer localization and also to distinguish between specific and nonspecific uptake of the radiotracer is to calculate the specificity or localization index. The dual isotope label study utilizing two different radionuclides, one labeled to the agent of interest and the other attached to a nonspecific localizing agent was introduced by Pressman and coworkers[177] to determine the localization index of the radiotracer in study. Localization index is calculated as:

$$\frac{\text{Tissue:blood ratio I-125 PNA}}{\text{Tissue:blood ratio I-131 nonspecific protein}}$$

The values obtained for the localization index calculated for uptake of ^{125}I-PNA in the TA3/Ha tumor was 1.19, 7.92 and 12.0 at 24, 48 and 72 hours post-injection of the tracer respectively as seen in table 4. These values obtained for the tumor were found to be significantly higher than most of the other tissues studied after 48 hours post-injection. The TA3/St tumor did not take up ^{125}I-PNA appreciably and

Table 3: Biodistribution of ^{125}I-PNA in A/J mice[1] bearing subcutaneous TA3/Ha and TA3/St tumors[2].

Tissues	24 hours	48 hours	72hours
Blood	0.59±0.22	0.10±0.05	0.05±0.02
Salivary glands	18.02±9.64	3.57±3.70	1.11±0.34
Spleen	0.92±0.40	0.25±0.13	0.16±0.04
Stomach*	5.02±1.78	0.67±0.58	0.27±0.08
GIT*	0.46±0.13	0.08±0.04	0.05±0.01
Kidneys	11.89±2.78	3.66±1.49	1.78±0.02
Muscle	0.33±0.25	0.05±0.02	0.03±0.01
Lungs	1.15±0.37	0.28±0.12	0.10±0.03
Liver	1.01±0.44	0.43±0.11	0.31±0.03
TA3/Ha tumor	1.92±0.17	1.16±0.24	0.63±0.18
TA3/St tumor	0.53±0.30	0.24±0.10	0.13±0.06

[1] $n = 3$

[2] Values are expressed as mean percent injected dose per gram of tissue ± standard deviation

* including contents

Table 4: Localization index of ^{125}I-PNA in A/J mice[1] bearing subcutaneous TA3/Ha and TA3/St tumors[2].

Tissues	24 hours	48 hours	72 hours
Salivary glands	2.89	4.78	16.67
Spleen	3.97	6.62	7.52
Stomach*	2.97	4.18	6.57
GIT*	2.87	3.06	4.14
Kidneys	5.20	3.58	3.51
Muscle	2.63	2.20	1.76
Lungs	4.28	5.74	3.41
Liver	2.83	5.45	5.75
TA3/Ha tumor	1.19	7.92	12.00
TA3/St tumor	1.86	2.3	*a*

[1] $n = 3$

[2] Values were calculated according to the formula introduced by Pressman and coworkers[274] using ^{131}I-F(ab')$_2$ as the [illegible] localizing agent.

* including contents

a = tumor tissue not sufficiently defined

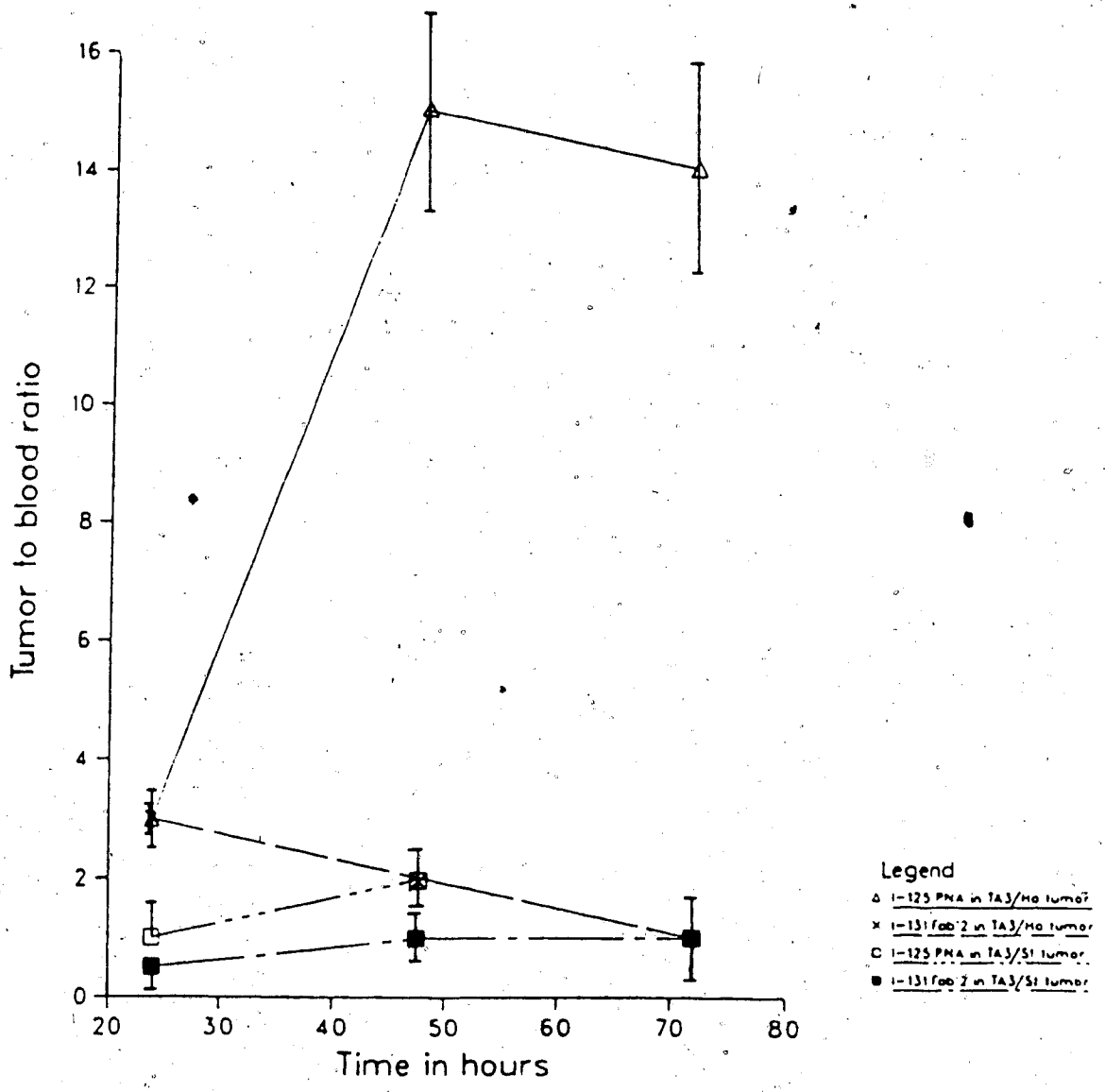


Figure 2 TA3/Ha tumor to blood ratio with ^{125}I-PNA in A/J mice bearing subcutaneous TA3/Ha and TA3/St tumors.

at times had localization index values that were lower than most other tissues. The results of this preliminary study and those of the *in vitro* studies prompted the further investigation of radiolabeled peanut lectin as a potential radioimaging agent for T-antigen bearing tumors. The TA3/Ha presents itself as a good animal tumor model as well as offering the possibility to study metastatic variants arising from a primary tumor.

Dosage determination of PNA

The results from the preliminary tissue biodistribution studies gave indication that I-125 labeled peanut lectin would be a potential radioimaging agent of tumors bearing T-antigenic specificities. To further investigate the potential utility as a radioimaging agent, the specificity of the radiopharmaceutical towards the target organ has to be determined as well as the amount of the injected dose taken up by other tissues. It would be useful to obtain enough accumulation of the radiotracer by the tumor to perform radioimaging studies. However, oversaturation of the tumor with the radiotracer could result in the excess radiotracer circulating in the blood, providing unnecessary radiation to the other organs as well as possibly decreasing the resolution of a scan due to increased background radioactivity. Therefore, it would be of practical use to determine a dosage of the radiotracer to administer to the patient which would result in maximum uptake of the radioimaging agent by the tumor with minimal uptake by other organs. It was found in the preliminary biodistribution study that PNA was rapidly cleared from the circulation and that few tissues retained PNA in such quantities as the TA3/Ha tumor.

In this study, a varying amount of radioiodinated PNA ranging from 0.1 μg to 60.0 μg was injected into TA3/Ha tumor bearing mice. Groups of these mice were injected intravenously with ^{125}I-PNA (0.1μg) and ^{131}I-BSA (0.1μg), ^{125}I-PNA (1.0μg) and ^{131}I-BSA (1.0μg), ^{125}I-PNA (10.0μg) and ^{131}I-BSA (10.0μg) and ^{125}I-PNA (60.0μg) with all injection doses in a total volume of 0.2 mL. Bovine serum albumin

was used in this study as a nonspecific tissue localization control. All the syringes were counted for radioactivity before and after injection to determine the actual injection dose. The mice were sacrficed twenty-four hours post-injection and the entire tissues of interest were excised from the animals. Samples of blood were withdrawn and the blood volume was extrapolated as 6.5% of the total body weight. A sample of the skeletal muscle was obtained from the hind thigh that was contralateral to the tumor site. The results of the study are presented in figure 3 showing the percentage of the injected dose taken up per gram of wet tissue, figure 4 which shows the TA3/Ha tumor to blood ratios for the various concentration of PNA administered and, figure 5 which illustrates the tumor to muscle ratios. From the results obtained as shown in the figures 3 to 5, it can be seen that there is an increase in tissue uptake of PNA with increasing doses for all the organs investigated including the tumor. However, when using the muscle and blood as non-target organs for comparison of target to non-target ratios of PNA localization (figures 4 and 5) in the tumor, an increase in the ratio is observed with decreasing dose. This implies that at lower doses there is a relatively higher tumor uptake of PNA in comparison with the other organs. This may be due to the saturation of available PNA receptor sites on the tumor cells by the higher doses of PNA administered thus allowing an excess of PNA to circulate around to the other organs. Using ^{131}I-BSA as the nonspecific localization control, it can be seen (table 5) that specific localization of PNA in the tumor decreases with increasing doses of protein administered. This implies that at higher doses of PNA administered, localization of the peanut lectin at the tumor site due to specific binding affinity of PNA for the T-antigenic receptors decreases, that is, nonspecific localization increases. An increase of PNA concentration in the blood with increasing dosage may be a contributing factor to the higher amounts of radioactivity observed in the other organs as well as at the tumor site. Figure 6 graphically shows the specific localization index that had been plotted from the data obtained for PNA at various administered doses.

Table 5: Localization index of varying amounts of ^{125}I-PNA in A/J mice[1] bearing subcutaneous TA3/Ha tumors at 24 hours post-injection[2].

Tissues	0.1μg	1.0μg	10.0μg
Salivary glands	20.21	13.07	13.25
Spleen	6.55	7.04	17.01
Stomach*	11.96	6.39	8.82
GIT*	5.81	4.73	4.69
Kidneys	41.51	112.17	286.50
Muscle	2.83	2.41	4.21
Lungs	4.95	10.21	14.21
Liver	5.12	3.35	3.26
TA3/Ha tumor	14.25	8.73	9.74

[1] $n = 5$

[2] Values were calculated using ^{131}I-BSA as the nonspecific localizing agent.

* including contents

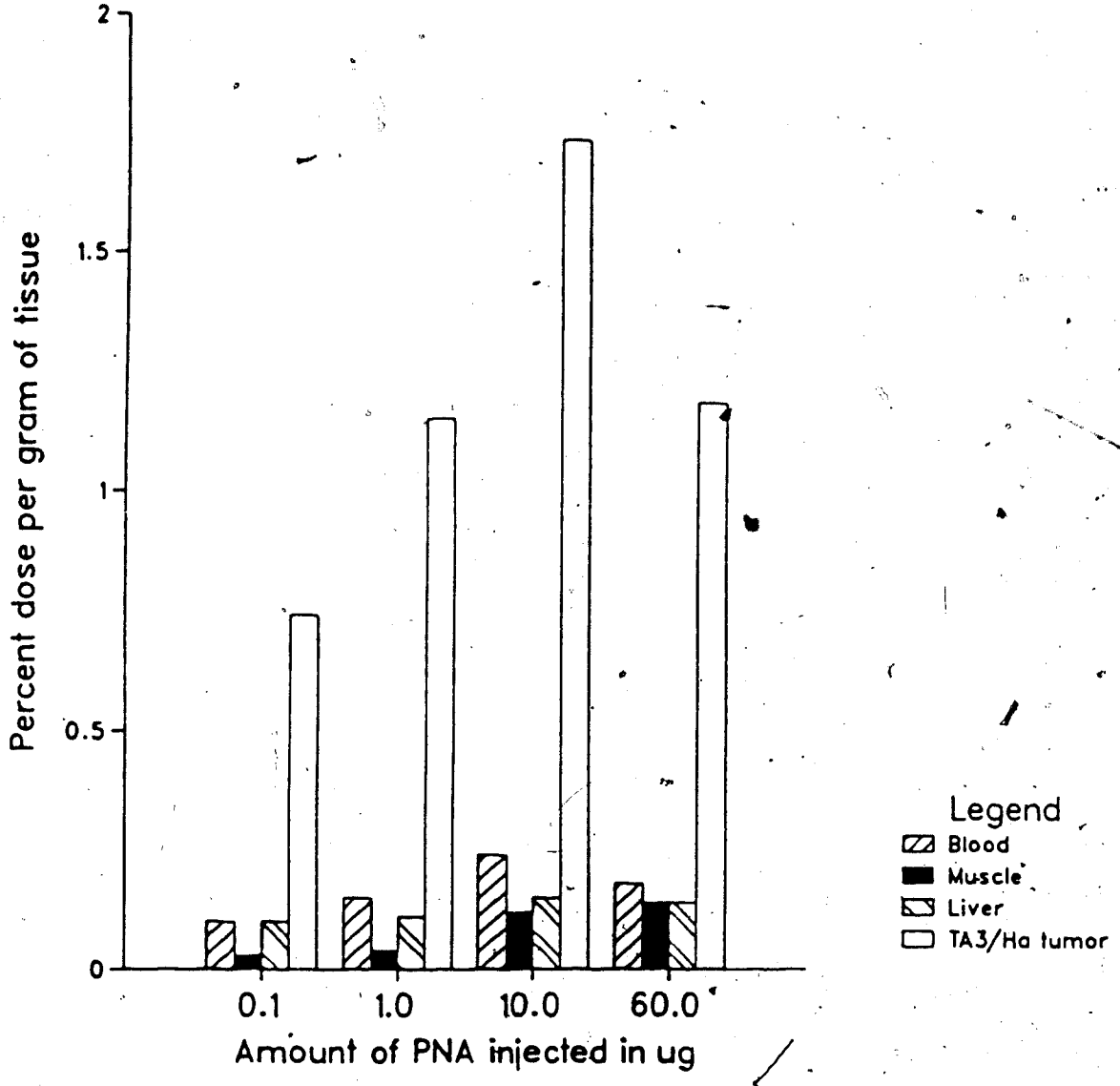


Figure 3 Effect of PNA dose on the 24 hour biodistribution of I-125 PNA in TA3/Ha tumor bearing mice

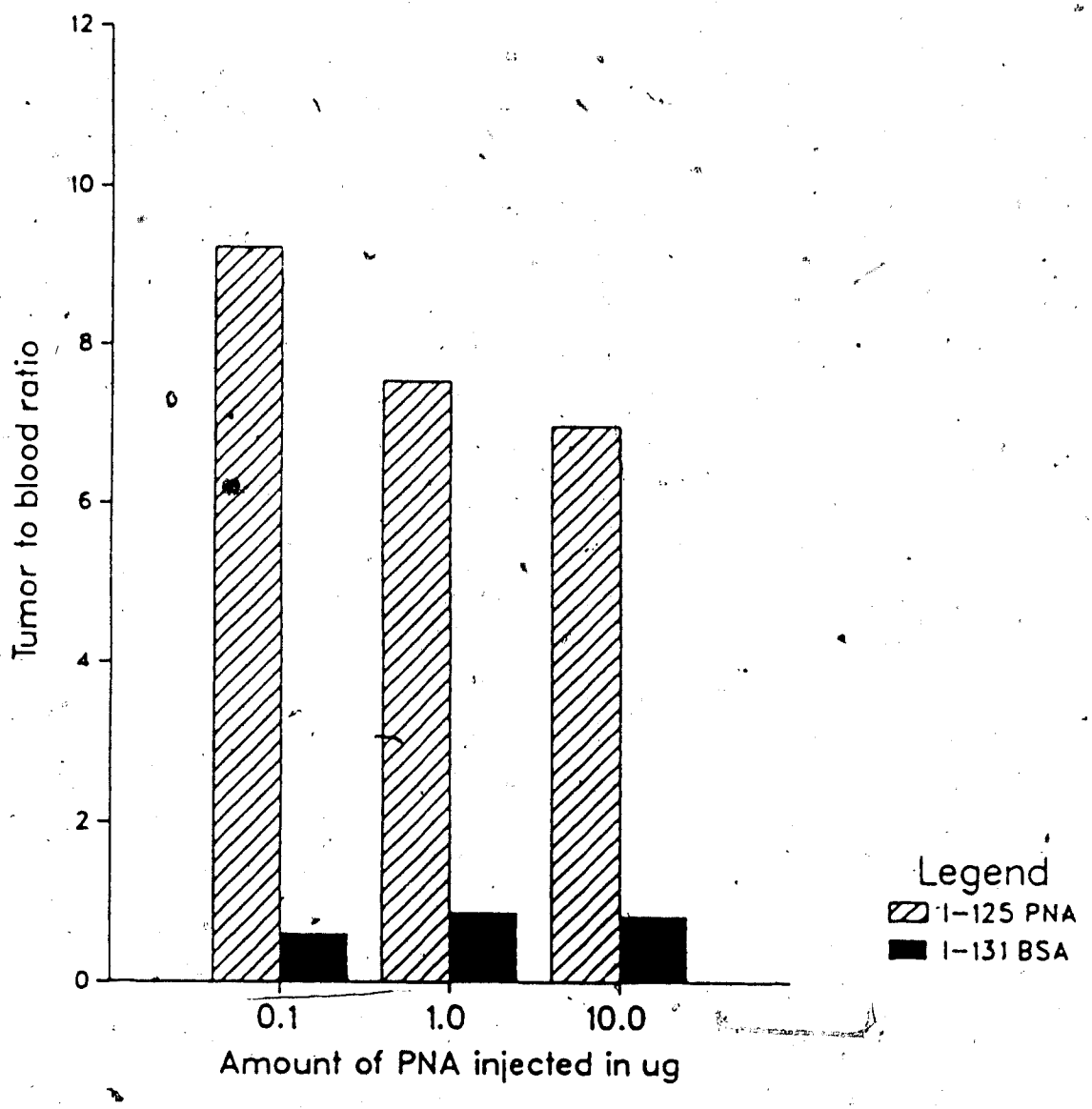


Figure 4 Effect of PNA dose on the tumor to blood ratios

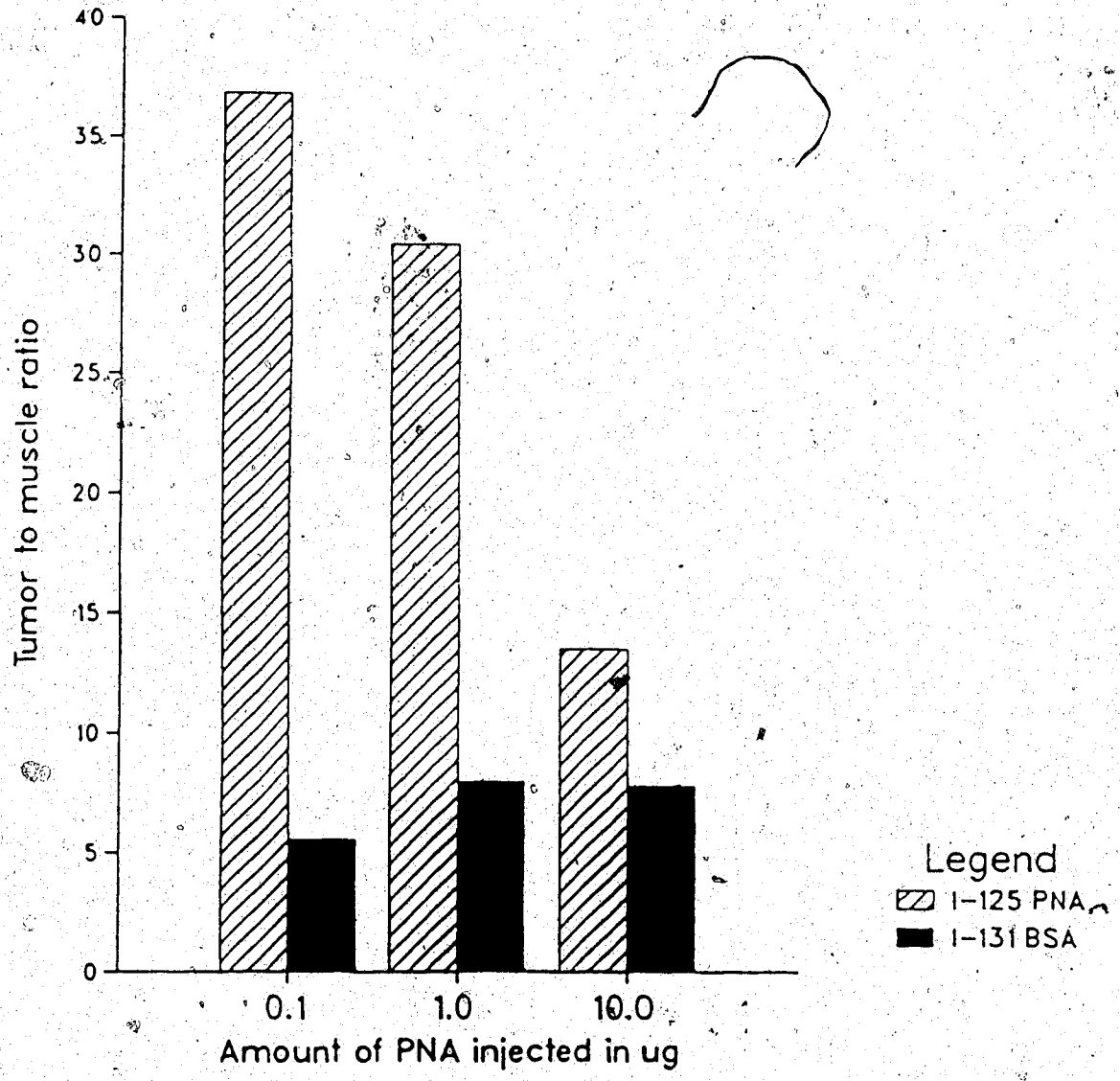


Figure 5 Effect of PNA dose on the tumor to muscle ratios

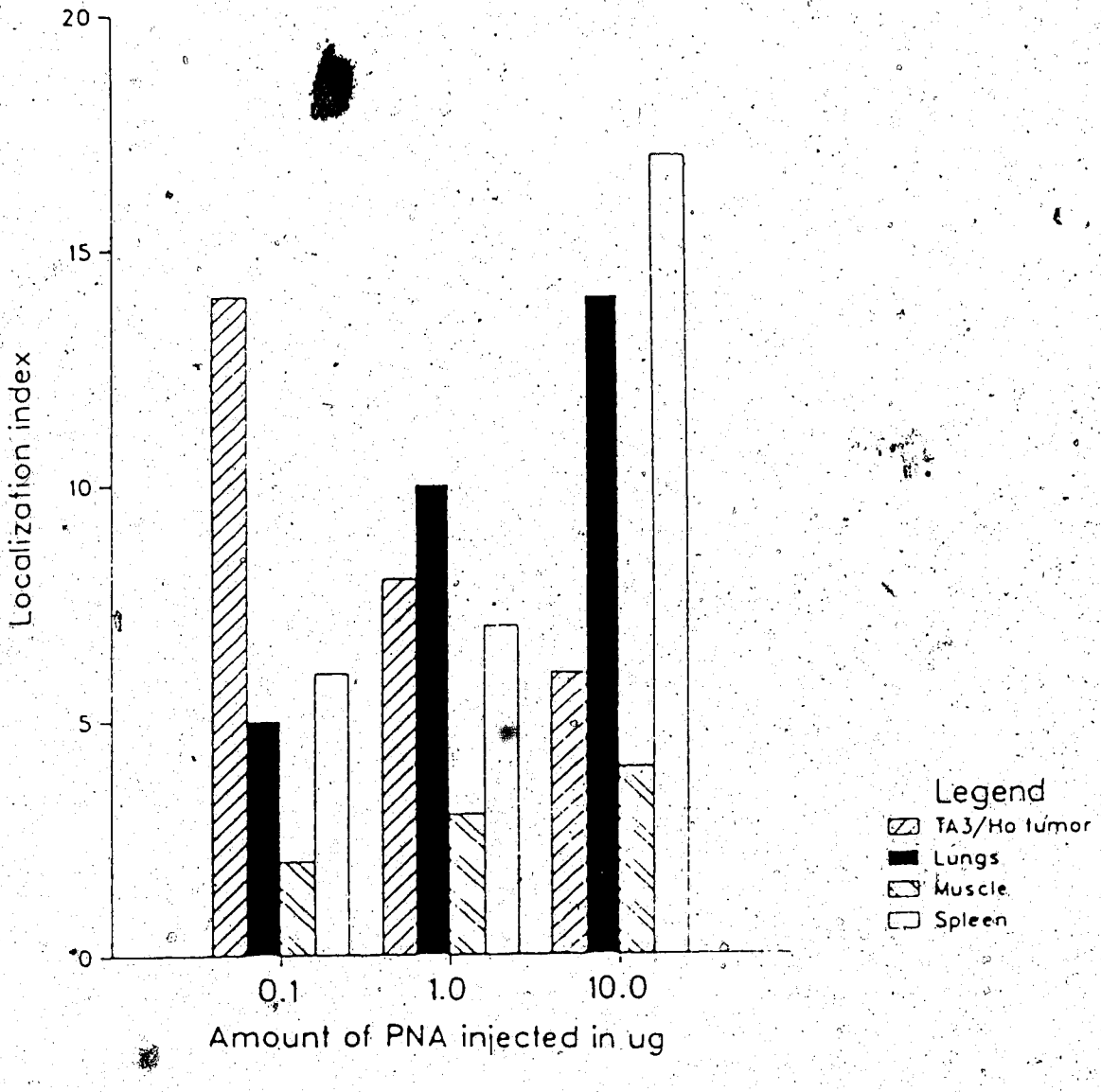


Figure 6 Localization index calculated for various PNA doses

Approximately 200 μL blood samples were obtained from the mice, counted for radioactivity, precipitated with 100 μL 25% TCA, washed twice with 1 mL PBS and the final precipitate and supernatant with the combined washes counted for radioactivity to determine the amount of free iodine in the circulation due to the process of deiodination *in vivo*. The results obtained for the amount of free radioiodide due to the *in vivo* breakdown of the radiotracer with the various groups of mice at the different adminstered doses are presented in figure 7. An increase in deiodination of ^{125}I-PNA was found with increasing doses of protein administered while the level of deiodination of ^{131}I-BSA remained about the same for all the different adminstered amounts.

From all the results obtained, it is surmised that the recommended amount of peanut lectin that should be administered to a 20 g mouse to obtain maximal tumor uptake with minimal nontarget organ accumulation of the radioiodinated PNA should be in the range of 0.1 μg to 1.0 μg. This dose range should give good tumor localization as seen from the high tumor to blood and tumor to muscle ratios while resulting in low background activity and also low radiation dose to the other organs. The rate of deiodination of the I-125 labeled peanut lectin is slower with the lower adminstered dose.

Biodistribution of PNA at various time intervals

The biodistribution of I-125 labeled peanut lectin was examined in mice bearing subcutaneous solid TA3/Ha tumors to determine the extent of localization of PNA in the various organs and tumor at various time intervals post-injection of PNA. This study would help determine the optimum time post-injection to perform scintigraphic imaging. In this study, ^{125}I-PNA and ^{131}I-nonspecific rabbit IgG F(ab')$_2$ fragments were injected intravenously into tumor bearing mice. The results of this study are presented in table 6 as the percentage of the injected dose per gram of tissue and table 7 as the percentage of the injected dose per whole organ. From the results it was calculated that there was a rapid total body clearance of radioiodinated PNA with

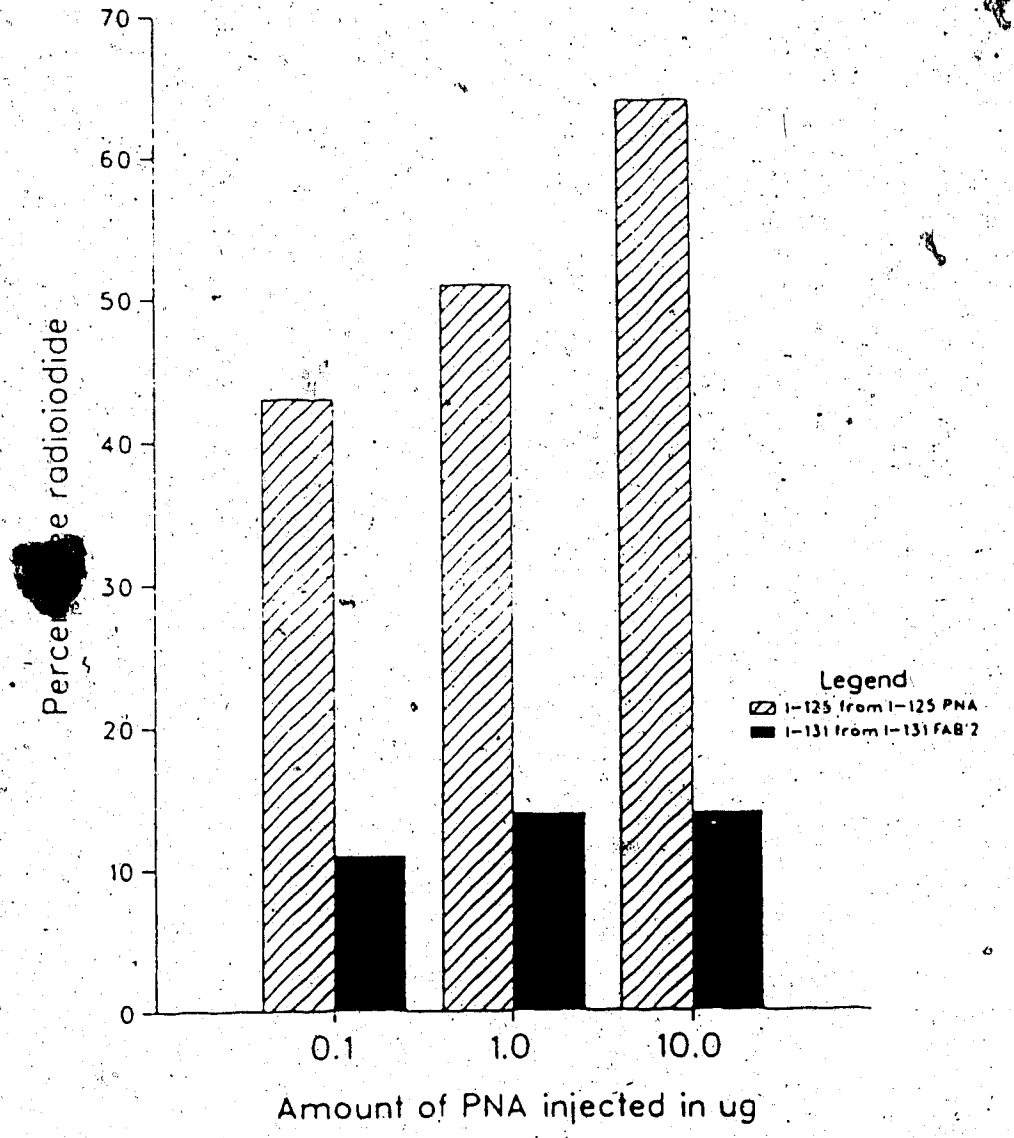


Figure 7 Levels of circulating free radioiodide at 24 hours following varying doses of I-125 PNA

73.9%, 69.1%, 9.7%, 2.6% and 2.5% of the injected dose remaining in the body at 3, 6, 24, 48 and 72 hours post-injection of PNA respectively. The data obtained from this study also shows that there is a relatively rapid clearance of the peanut lectin from the various tissues except for the kidneys and the tumor. Blood levels of the radiolabeled peanut lectin was also seen to decrease rather rapidly with 7.4 ± 1.7, 8.1 ± 2.6, 0.83 ± 0.4, 0.04 ± 0.01, and 0.03 ± 0.01 percent of the injected dose ± standard deviation in the blood remaining at 3, 6, 24, 48 and 72 hours post-injection. A considerable amount of background activity in the various organs was still apparent at 6 hours post-injection with ^{125}I-PNA concentration in some organs higher than that of the tumor on a per gram as well as on the whole organ basis. This changed, however, after 24 hours post-injection and the kidneys were the only organs that had higher concentrations of radioactivity than the tumor. All the other organs examined except for the stomach and salivary glands had very low amounts of ^{125}I-PNA at 24 hours post-injection. Organ to blood ratios were found to be relatively insignificant for most of the tissues at 24 hours post-injection except for the tumor, kidneys, stomach and salivary glands. The rapid blood clearence of ^{125}I-PNA and the prolonged retention of the peanut lectin by the tumor resulted in tumor to blood ratios of 2.3:1, 7:1 and 55:1 at 6, 24 and 48 hours post-injection respectively as shown in figure 8. Another non-target tissue taken was the muscle, and the plot of tumor to muscle ratio in figure 9 gives values of 6:1, 33:1 and 77:1 at the time periods 6, 24 and 48 hours post-injection respectively. With these results, it would appear that radioimaging of the TA3/Ha solid tumors in the mice would provide a relatively good delineation as a result of the high tumor to blood and tumor to muscle ratios combined with the low background activity contributed by most of the other organs except for the kidneys. Analysis of the plasma samples obtained from the mice at various time periods post-injection to determine the rate of deiodination of ^{125}I-PNA resulted in the observation of a rapid breakdown of the label *in vivo* thus leading to production of free radioiodide in the circulation. The amount of

Table 6: Biodistribution of ^{125}I-PNA in A/J mice[1] bearing subcutaneous TA3/Ha tumors at various time intervals[2,3].

Tissues	3 hours	6 hours	24 hours	48 hours	72hours
Blood	5.74±1.27	5.75±1.34	0.67±0.44	0.03±0.01	0.02±0.01
Salivary glands	21.73±6.93	29.07±1.22	5.18±1.82	2.25±0.38	1.42±0.50
Spleen	4.64±0.63	3.97±0.60	0.55±0.30	0.15±0.05	0.22±0.10
Stomach*	15.67±3.30	21.42±4.37	4.47±1.94	0.20±0.03	0.27±0.15
GIT*	4.04±0.54	3.97±0.36	0.45±0.27	0.06±0.03	0.06±0.03
Kidneys	136.69±35.54	99.62±24.61	9.77±5.48	2.44±0.55	2.83±1.66
Muscle	2.24±0.69	1.98±0.63	0.14±0.09	0.02±0.00	0.01±0.00
Lungs	10.07±1.51	11.17±3.68	2.06±0.94	0.19±0.08	0.12±0.06
Liver	3.34±0.29	3.56±0.31	0.96±0.41	0.41±0.05	0.46±0.12
TA3/Ha tumor	13.01±4.97	13.25±4.69	4.39±1.72	1.54±0.52	1.52±0.53

[1] $n = 8$

[2] Values are expressed as the mean percent injected dose per gram of tissue ± standard deviation.

[3] 150 kBq ^{125}I-PNA (1.0μg) injected intravenously.

* including contents

Table 7: Percent injected ^{125}I-PNA dose per whole organ in A/J mice[1] bearing subcutaneous TA3/Ha tumor[2].

Tissues	3 hours	6 hours	24 hours	48 hours	72hours
Blood	7.45±1.65	8.08±2.55	0.83±0.41	0.04±0.01	0.03±0.01
Salivary glands	2.50±0.47	4.71±0.50	0.74±0.16	0.35±0.04	0.18±0.09
Spleen	0.59±0.11	0.45±0.12	0.08±0.04	0.03±0.01	0.02±0.01
Stomach*	6.77±0.62	9.43±3.97	1.92±0.72	0.23±0.11	0.09±0.06
GIT*	7.73±1.70	7.73±1.14	0.75±0.32	0.15±0.09	0.12±0.06
Kidneys	39.68±3.56	30.66±3.20	2.63±0.81	0.85±0.11	0.64±0.22
Lungs	1.37±0.26	1.53±0.49	0.26±0.11	0.03±0.01	0.02±0.01
Liver	4.05±0.64	4.48±0.12	1.02±0.20	0.62±0.02	0.46±0.10
TA3/Ha tumor	3.04±2.00	1.58±0.18	1.43±0.66	0.33±0.14	0.91±0.76

[1] $n = 8$

[2] Values are expressed as mean percent injected dose ± standard deviation per entire organ at various time intervals following intravenous 150 kBq ^{125}I-PNA (1.0μg).

* including contents

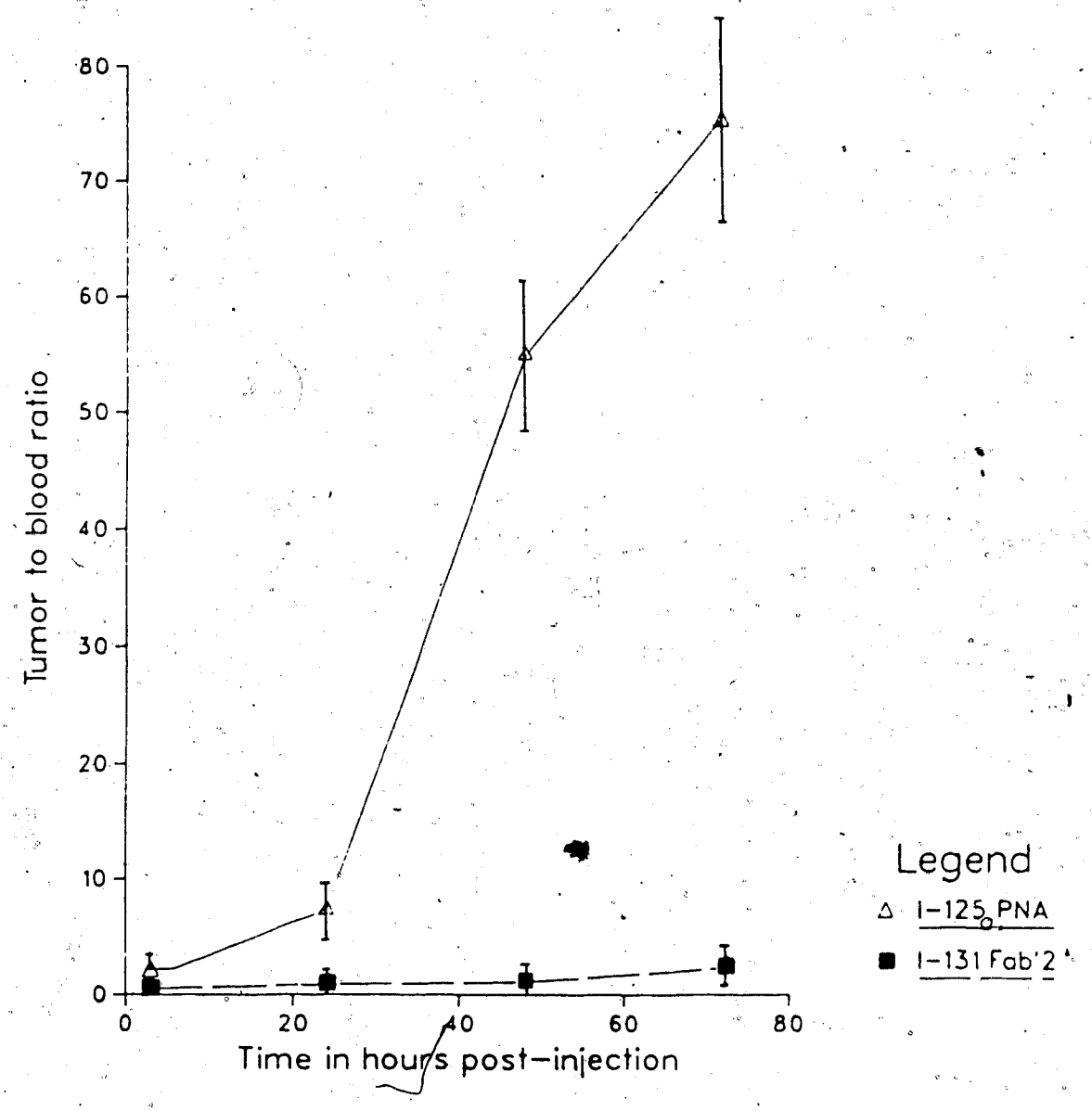


Figure 8 Tumor to blood ratio of ^{125}I-PNA

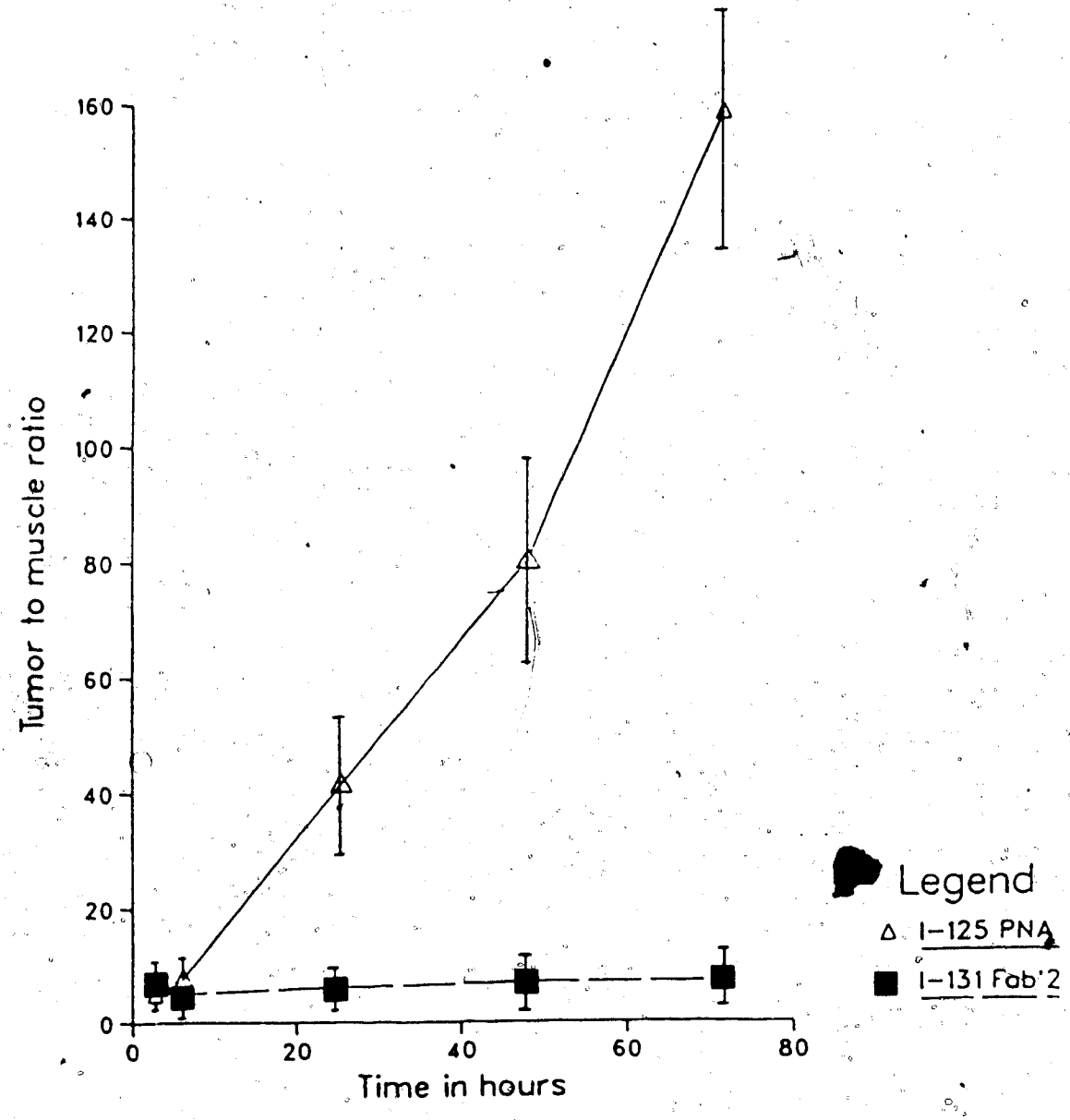


Figure 9 Tumor to muscle ratio of ^{125}I-PNA

free radioiodide found in the circulation as determined by the TCA precipitation of the plasma samples were 39.2 ± 10.7%, 52.5 ± 9.9%, 59.7 ± 17.3%, 65.2 ± 6.4% and 74.5 ± 8.6% of the total amount of radioactivity in the plasma samples at 3, 6, 24, 48 and 72 hours post-injection respectively. The high amount of circulating free iodide could be a contributing factor to the high levels of radioactivity observed in the kidneys, stomach and salivary glands all of which are known routes for iodide excretion.

The uptake of PNA by the stomach could also be partly due to some specific PNA binding as histochemical studies have demonstrated the presence of PNA binding sites in the tissue sections of the stomach and esophagus in the mouse[79,110]. It could also be possible that the presence of galactosyl containing glycoproteins found in the salivary mucins bound the peanut lectin thus leading to the high amounts of radioactivity observed in the salivary glands. The large amounts of radioactivity seen in the kidneys could not be totally due to the presence of free radioiodide alone. It was found that the urine samples obtained from these animals contained the radioactivity bound to a protein since a significant amount of radioactivity in the urine could be precipitated by 25% TCA. Urine samples obtained and precipitated by TCA revealed that there was about 86% of the total radioactivity in the urine protein bound at 30 minutes post-injection, 46% bound at 6 hours and 22% at 24 hours post-injection of ^{125}I-PNA. Incubating the urine samples with asialo GM-1 synsorb revealed that a significant amount of the radioactivity bound to the immunoadsorbent implying that the active intact form of PNA is excreted out into the urine by the kidneys. It is, therefore, possible that the kidneys possess available binding sites for PNA *in vivo*.

To determine the specificity of the PNA binding to the tumor, the dual isotope experiment utilizing ^{125}I-PNA and ^{131}I-nonspecific rabbit IgG F(ab')$_2$ fragments was used based on the idea proposed by Pressman and coworkers[277]. Nonspecific rabbit IgG F(ab')$_2$ fragments of molecular weight about a 100 000 daltons were used as an indicator of nonspecific localization on the basis of its similar molecular weight to the

peanut lectin and also due to a lack of a suitable inactive peanut lectin for comparison. The results of the localization index calculated for the various time intervals of the biodistribution study are presented in table 8. At 24 hours post-injection, the calculated localization index was 6.06 for the salivary glands, 3.3 for the stomach, 8.8 for the kidneys and 8.5 for the tumor. At 48 hours only the tumor and the kidneys had significant localization index values with 37.2 for the kidneys and 49.5 for the tumor. Hence, only the tumor and the kidneys could be said to demonstrate high amounts of peanut lectin specific localization. The amount of radioactivity remaining in the whole body of the animals was determined at the various times of sacrifice. This then made it possible to determine the percentage of the total remaining injected dose which was contained per gram of tissue at the different time periods post-injection. This information would relate the relative rate of the clearance/retention of the radiolabeled peanut lectin by the various tissues. Figure 10 illustrates the data of the percent of the total body remaining injected dose found per gram of the tissue at the various time intervals. It can be seen from figure 10 that the retention of the radiolabeled peanut lectin was significant by the kidneys and the tumor. Most of the other tissues did not show any significant retention of the labeled protein. The high amounts of radioactivity observed in the kidneys at the earlier time periods is probably due to the excretion of the labeled PNA into the urine as evidenced by the precipitable amount of radioactivity in the urine samples and the absoption of a significant amount of radioactivity to the asialo GM1-sorb. Also, the levels of free circulating radioiodide at the earlier time periods were low, hence contribution of radioactivity seen in the kidneys by free radioiodide at this time was low. At later time periods, however, there were higher amounts of free circulating radioiodide which probably contributed to the increased accumulation of radioactivity in the kidneys. TCA precipitation of the urine at 24 hours revealed that only about 22% of the total radioactivity was protein bound and asialo GM1-sorb absorption found that about 13% of the total radioactivity bound to the

Table 8: Localization index of ^{125}I-PNA in A/J mice[1] bearing subcutaneous TA3/Ha tumors at various time intervals[2].

Tissues	3 hours	6 hours	24 hours	48 hours	72hours
Salivary glands	7.12	3.45	6.06	13.79	35.42
Spleen	4.13	2.80	0.27	11.18	7.29
Stomach*	0.71	3.24	2.35	11.62	6.70
GIT*	5.16	3.00	2.37	8.53	4.46
Kidneys	13.00	11.07	8.80	37.16	20.24
Muscle	4.20	2.39	1.46	4.18	1.52
Lungs	3.46	4.07	5.82	8.34	3.50
Liver	2.19	1.73	3.79	21.74	13.52
TA3/Ha tumor	4.36	3.68	8.59	49.50	25.29

[1] $n = 8$

[2] Values were calculated using ^{131}I-F(ab'), as the nonspecific localizing agent.

* including contents

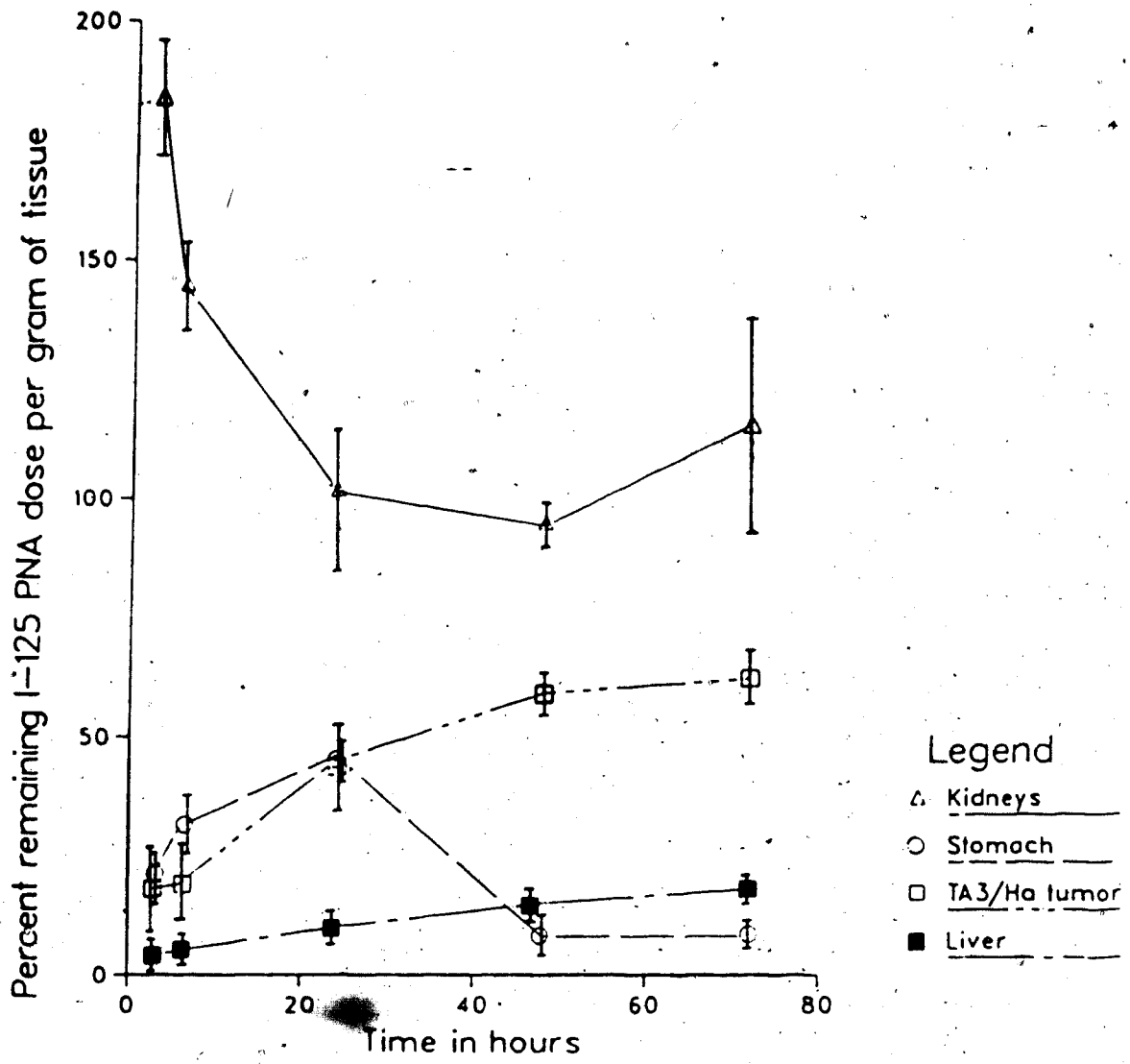


Figure 10 Percent uptake of the remaining ^{125}I-PNA dose per gram of tissue.

synthetic T-hapten. The tumor, however, exhibited a retention characteristic of the radioiodinated peanut lectin continually throughout the biodistribution study. The percentage of the remaining injected dose per gram of tumor tissue was calculated to be 17.6%, 19.2%, 45.3%, 58.9% and 61.5% at 3, 6, 24, 48 and 72 hours post-injection of ^{125}I-PNA respectively. Based on all these results obtained in this time interval biodistribution study, it appears that the potential exists for radiolabeled peanut lectin to provide a high localization index and good delineation of the tumor by gamma scintigraphy 24 hours post-injection of radioiodinated peanut lectin.

Whole body scintigraphic imaging

To illustrate the potential usefulness of radiolabeled peanut lectin as a radioimaging agent of T-antigen bearing tumors, mice with solid TA3/Ha tumors in their right flank were injected with ^{125}I-PNA and scintigraphic images were taken. The tumors in the mice were allowed to grow to about 150 mg in size before the study was initiated. At various time intervals of 6, 24, 48 and 72 hours post-injection of the radiolabeled peanut lectin, the mice were lightly anesthesized and restrained in a prone position while images were collected from the posterior view using a Searle Pho/Gamma IV camera with a pinhole collimator. The aquisition data was stored on a floppy disk with the aid of the ADAC CAM II clinical aquisition processing unit. Results of the study are shown in the following figures 11 to 14. Each of the images obtained were accumulated to a total of 50,000 counts. In figure 11, a rather diffuse whole body image is seen with the image taken at 6 hours post-injection of PNA. This image was quite expected as the biodistribution study performed indicated that at this time period after injection, about 69% of the injected dose was still in the body and most of the organs had high accumulations of radioactivity. A clearer image is obtained, figure 12, at 24 hours post-injection where there is only about 9.6% of the injected dose remaining in the whole body and most of the radioactivity has cleared from the organs except for the tumor, kidneys and salivary glands. There were some areas of radioactivity seen in the area above the right kidney which could be accumulation of

Figure 11 Whole body scintigraphic image of a mouse bearing TA3/Ha subcutaneous tumor; 6 hours post-injection of 2 MBq ^{125}I-PNA

Figure 12 Whole body scintigraphic image of a mouse bearing TA3/Ha subcutaneous tumor; 24 hours post-injection of 2 MBq ^{125}I-PNA

Figure 13 Whole body scintigraphic image of a mouse bearing TA3/Ha subcutaneous tumor; 48 hours post-injection of 2 MBq ^{125}I-PNA

Figure 14 Whole body scintigraphic image of a mouse bearing TA3/Ha subcutaneous tumor; 72 hours post-injection of 2 MBq ^{125}I-PNA

PNA in the stomach 24 hours post-injection. Biodistribution data indicated a high blood clearance of the radiolabeled peanut lectin with 0.67% and 0.03% of the injected dose per gram of blood at 24 and 48 hours post-injection respectively. Furthermore, after 24 hours post-injection, only the kidneys and the tumor showed any significant retention of radioactivity (figure 12). As evidenced by the figures 13 and 14, very little background activity could be visualized at later time periods. A clear delineation of the tumor could be seen at 48 and 72 hours post-injection of the peanut lectin as demonstrated by the intense radioactivity localization at the known anatomical site of the tumor. Hence, the scintigraphic images of the tumor bearing mice complements the biodistribution data obtained for the mice at various time periods of PNA localization *in vivo*. The high tumor to blood [illegible] to muscle ratios obtained with the radioiodinated peanut lectin and the clear scintigraphic images of the tumor in the mice further potentiates the usefulness of the peanut lectin as a T-antigen tumor localizing and imaging agent. These clear images with good delineation of the tumor in the mice were obtained without the need of any background subtraction or computer manipulation of the acquisition data/image.

Many PNA binding sites have been identified in various murine tissue sections using immunohistochemical methods with labeled PNA[79,80]. Most of the positive PNA binding sites were identified especially in secretory bodies, golgi zones and apical cell surfaces of the various cell types[79,80]. However, most of these sites are organelles within a cell and are not readily accessible to PNA in *in vivo* circulation. The process of tissue sectioning may reveal sites that are poorly vascularized or immunologically previleged[51,100] and it has also been found that the processing of tissue sections with certain chemicals such as formalin may cause some process of autolysis resulting in the exposure of normally cryptic T antigenic structures[110] resulting in a positive PNA binding reaction. Biodistribution data and whole body gamma scintigraphic images of a mouse would not reveal such PNA binding sites but only PNA reactive sites that are accessible *in vivo* such as the kidneys and the subcutaneously transplanted TA3/Ha tumor.

Whole body clearance of the peanut lectin

The whole body clearance of ^{125}I-PNA from the animal was determined to check whether the TA3/Ha tumor and its shed epiglycanin molecules would have any effect on the retention of the peanut lectin in the animal. Whole body clearance was measured at various time periods post-injection of the radioiodinated PNA. Three groups of mice were studied; one group was bearing a small solid form of the TA3/Ha tumor in their right flank, one group was bearing large amounts of the TA3/Ha tumor intraperitoneally in the ascites form and the last group of mice were normal control animals. Figure 15 illustrates the results of this study indicating that the mice bearing the tumor in the ascites form had a much longer retention time than either the controls or the mice bearing the solid tumor. The cause of this large difference in retention time of radioiodinated peanut lectin is most probably due to the presence of large quantities of the tumor cell surface epiglycanin. Epiglycanin has been found to express T-antigenic carbohydrate structures in 60% of its total oligosaccharide chains[246]. The high molecular weight epiglycanin is shed into the circulation and ascites fluid by the tumor cells. Purified epiglycanin fractions obtained from the ascites fluid of the mice bearing the TA3/Ha tumor in the ascites form has been found to bind avidly to the peanut lectin. The epiglycanin molecule has a high molecular weight of approximately 500 000 daltons, and being such a large structure, will have restricted movement from the peritoneal cavity to the circulation. This could account for the high retention of radioactivity seen for the ascites TA3/Ha tumor bearing mice as the ^{125}I-PNA bound epiglycanin complex in the ascites fluid would tend to remain in the peritoneal cavity. The solid tumor bearing mice show a retention of the radiolabeled peanut lectin similar to the control mice. The solid TA3/Ha tumor has been seen to be very well vascularized and epiglycanin molecules have been known to be shed into the circulation. However, the retention of radioactvity following intravenous injection of ^{125}I-PNA was not significantly different in the mice bearing subcutaneous TA3/Ha tumors as compared to the controls. It appears then, that the small (150-200 mg) solid form of the TA3/Ha tumor and its associated cell surface epiglycanin does not contribute significantly to the whole body retention

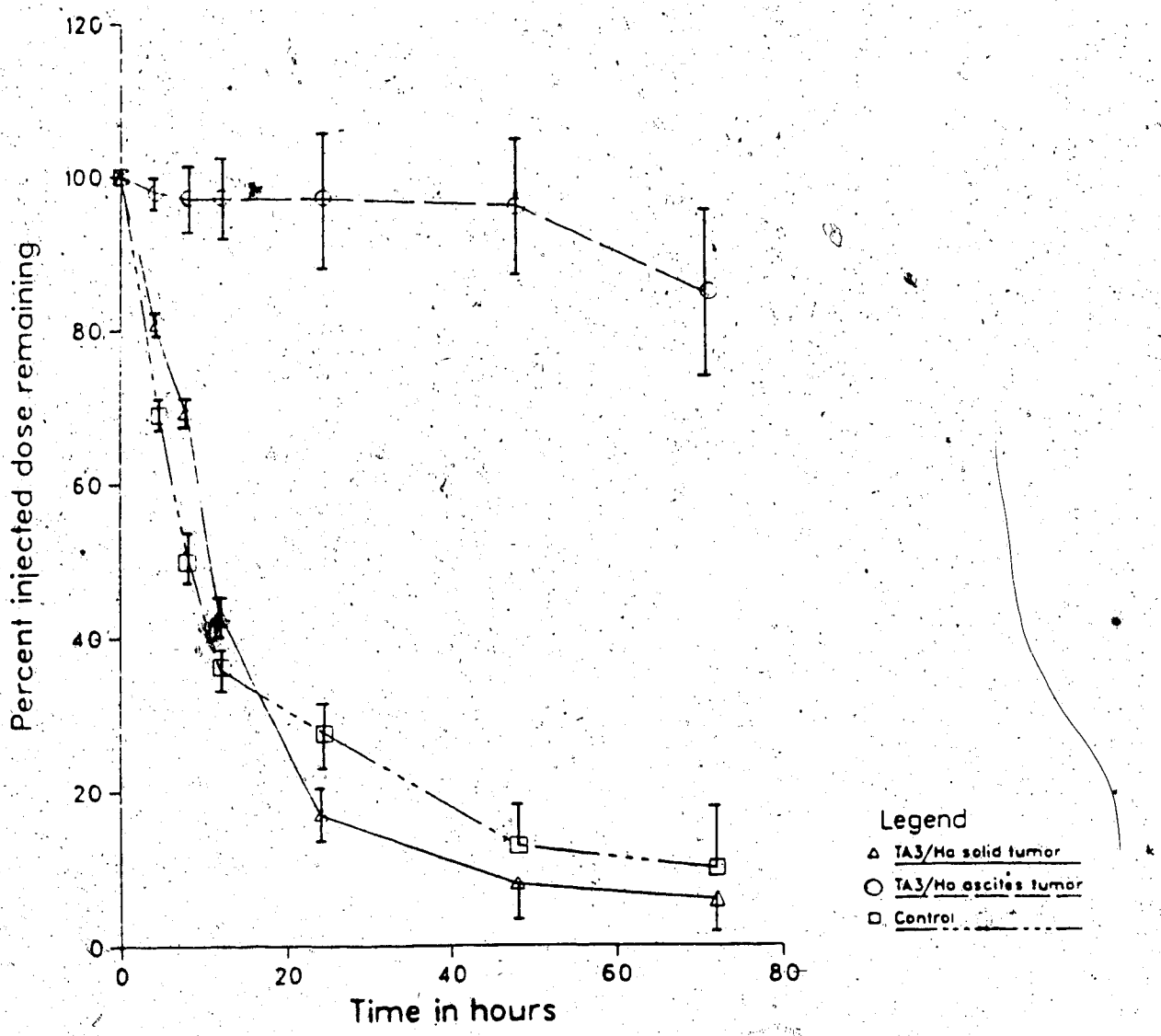


Figure 15 Whole body retention of I-125 PNA.

of the radiolabeled peanut lectin except in the tumor itself.

Biodistribution of $Na^{125}I$

The rapid deiodination of radiolabeled peanut lectin *in vivo* releasing free radioiodide into the circulation may have contributed to the high uptake of radioactivity in some of the organs. This high level of free radioiodide in the circulation also led to the concern that the highly vascularized tumor may in fact be taking up free radioiodide and that the high amounts of radioactivity localized in the tumor may be just a large concentration of free radioiodide. Therefore, to determine the extent of radioiodide concentration in the tumor and the other tissues due to the rapid breakdown of the label *in vivo*, radioiodide in the form of $Na^{125}I$ (150 kBq) was injected intravenously into TA3/Ha tumor bearing mice. As previously, KI was added to the drinking water commencing 3 days prior to the study. At 6, 24 and 48 hours post-injection of the radioiodide solution, the mice were sacrificed and the various tissues dissected, weighed and counted for radioactivity. The results of the study are given in table 9 in which the values are given as the percentage of the injected dose per gram of tissue. The organs of interest here are the kidneys, salivary glands, stomach and tumor as these are the organs that showed high amounts of radioactivity localization with the radioiodinated peanut lectin. The results (table 9) indicate high amounts of radioactivity concentration in the stomach and salivary glands while the kidneys, tumor and the rest of the tissues showed less accumulation. Uptake of radioactivity by the stomach, including contents, was measured at 43.6, 0.7 and 0.075 percent of the injected dose per gram of tissue at 6, 24 and 48 hours post-injection and the salivary glands were found to have 35.0, 0.48 and 0.054 percent of the injected dose per gram of tissue at the same time periods. To determine the amount of radioactivity in the tissue that is due to the levels of radioiodide in the blood, tissue to blood ratios for the various tissues were calculated (table 10) and the results obtained showed that the salivary glands and stomach were the only organs to have significantly high ratios. The rest of the tissues had ratios of less than one for most of the time periods studied. In comparing the results obtained in this study

Table 9: Biodistribution of $Na^{123}I$ in A/J mice[1] bearing subcutaneous TA3/Ha tumor[2].

Tissues	6 hours	24 hours	48 hours
Blood	8.47±1.69	0.07±0.02	0.01±0.00
Salivary glands	35.05±9.34	0.48±0.23	0.06±0.02
Spleen	3.78±0.60	0.04±0.01	0.01±0.00
Stomach*	43.60±9.29	0.73±0.21	0.08±0.03
GIT*	4.47±0.85	0.05±0.01	0.01±0.00
Kidneys	6.11±1.12	0.05±0.01	0.01±0.00
Muscle	1.80±0.38	0.02±0.00	0.01±0.00
Lungs	6.02±1.06	0.06±0.01	0.02±0.01
Liver	3.94±0.87	0.05±0.02	0.01±0.00
TA3/Ha tumor	5.69±1.23	0.06±0.01	0.04±0.03

[1] $n = 8$

[2] Values are expressed as the mean percent injected dose ± standard deviation per gram of tissue at various times after intravenous $Na^{123}I$ (150 kBq).

* including contents

Table 10: Tissue to blood ratio of $Na^{123}I$ in mice[1] bearing subcutaneous TA3/Ha tumor[2].

Tissues	6 hours	24 hours	48 hours
Blood	1.00±0.00	1.00±0.00	1.00±0.00
Salivary glands	4.34±1.60	7.56±3.34	5.36±1.63
Spleen	0.45±0.06	0.71±0.28	0.95±0.19
Stomach	5.39±2.06	12.43±6.56	7.53±2.29
GIT	0.54±0.11	0.77±0.14	1.16±0.31
Kidneys	0.73±0.12	0.86±0.21	1.23±0.14
Muscle	0.21±0.03	0.30±0.09	0.95±0.41
Lungs	0.72±0.08	0.98±0.31	2.11±0.53
Liver	0.47±0.06	0.80±0.36	1.37±0.33
TA3/Ha tumor	0.68±0.13	0.92±0.26	3.96±2.48

[1] $n = 8$

[2] Values are expressed as the mean ratio ± standard deviation of radioactivity per gram of tissue compared to 1 gram of whole blood at various time periods following intravenous injection of 150 kBq $Na^{123}I$.

to that of the biodistribution study with ^{125}I-PNA, a distinct difference of accumulated radioactivity is observed for the kidneys and the tumor. In this study, the uptake by these two tissues are low whereas there is a significant uptake of radioactivity with the radiolabeled peanut lectin.

A plot of the percentage of the injected Na^{125}I dose per gram of tissue based on the total remaining radioactivity in the body is illustrated in figure 16 for the kidneys, stomach, salivary glands and tumor. The salivary glands and the stomach demonstrate a much higher amount of radioactivity retention than the kidneys and the tumor. This observation may help to explain the high levels of radioactivity observed in the stomach and salivary glands with radioiodinated PNA. As can be seen from the results obtained , the large proportion of the free radioiodide taken up by the stomach and salivary glands may help contribute to the high levels of radioactivity observed in the biodistribution study with PNA. More importantly, the results of this study demonstrate that the accumulation of radioactivity in the tumor seen in the biodistribution study with PNA has very little, if any, contribution from the free radioiodide generated by the rapid deiodination of the radiolabel *in vivo*. The tumor to blood ratio of radioiodide following injection of Na^{125}I showed that most of the radioactivity observed in the tumor was due to the blood borne radioiodide in the highly vascularized tumor. The plots of percentage of the remaining dose per gram of tumor for the two different biodistribution studies (figures 10 and 16) illustrate the difference in retention characteristics of the peanut lectin and radioiodide in the tumor. As for the kidneys, the results obtained in this study differ significantly from those in the PNA biodistribution study. It is known that a large fraction of the free circulating iodide is filtered through the kidneys[251]. This is the case seen in this Na^{125}I study but not with the I-125 labeled peanut lectin. A high level of radioactivity is initially observed in the kidneys but this is cleared very rapidly with the Na^{125}I but not with ^{125}I-PNA. The plots of percentage of the remaining injected dose per gram of kidney tissue for the two studies demonstrate the difference in retention characteristics of the kidneys for the two radiotracers. The results of these two different biodistribution studies indicate that the kidneys

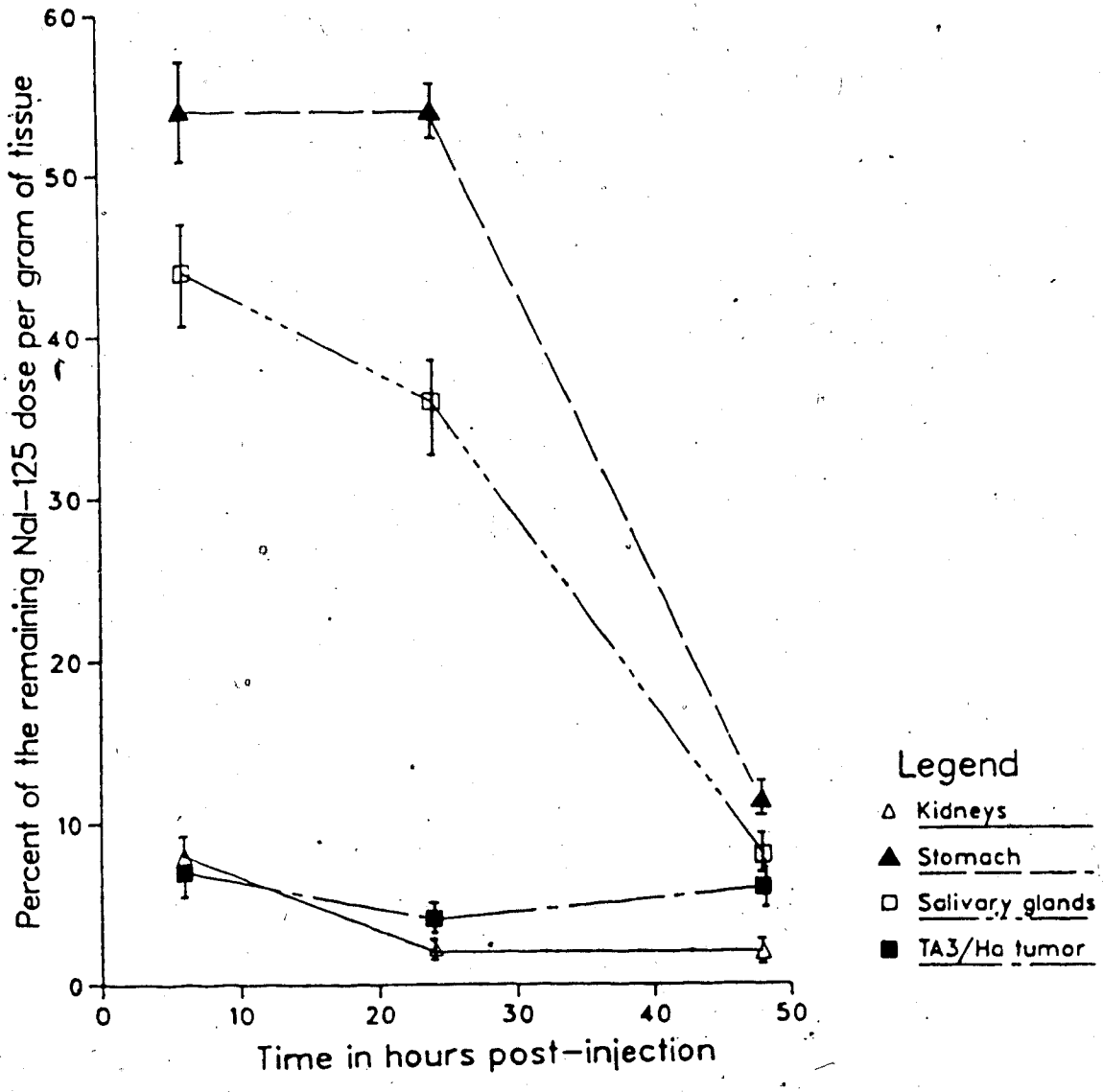


Figure 16 Percent uptake of the remaining $Na^{125}I$ dose/gram of tissue.

do retain PNA. The possibilty also exists that the epiglycanin shed from the surface of the TA3/Ha tumor into the circulation may complex with the radioiodinated peanut lectin and subsequently be deposited in the kidneys resulting in the high kidney accumulation of radioactivity, however it has been found that high kidney accumulation of radiolabeled peanut lectin also occurs in non-tumor bearing mice. Histochemical studies performed with FITC- and horse radish peroxidase-PNA on frozen kidney sections of mice revealed that the Bowmann's capsule, proximal tubules, distal tubules and the membrane and cytoplasm of the collecting ducts have PNA receptor sites[79,80,101]. Urine samples obtained from normal and tumor bearing mice that had been injected with the peanut lectin were found to contain the intact peanut lectin in an active form that binds to asialo GM1 synsorb. Thus, the kidney tubules may be involved in a unique mechanism for binding and excreting the peanut lectin.

Detection of TA3/Ha metastatic tumor variants

The availability of metastatic tumor variants that grow in specific organs when injected intravenously into the animals allows the study and evaluation of tumor localizing agents as potential radioimaging agents for metastatic tumor spread. The use of solid and well-vascularized transplanted tumors intramuscularly or subcutaneuosly has been found not to provide a sufficiently demanding tumor model to evaluate the potential of a radiolocalizing agent in detecting metastases[278]. It may be possible that with tumors which have been transplanted intramuscularly or subcutaneously a vascular supply exists that is different than that of the metastatic tumors arising from them[278]. Various conditions such as the anatomic location of the tumor, the blood supply to the tumor, the behaviour of the agent in the systemic circulation, and the structure and permeability of the tumor vasculature[278] influence the localization and retention of an agent administered intravenously to animals bearing metastatic tumors. The presence of antigen-positive tumor cells and the properties of the target antigen will further affect the retention of the antigen-binding agents[278].

Selection of the TA3/Ha lung and liver metastatic tumor variants

The process of selecting the TA3/Ha lung and liver specific metastatic tumor variants was performed in the laboratory of Dr. B.M. Longenecker, Department of Immunology, University of Alberta according to the method described by Fidler[41]. Strain A/J mice which had been injected intravenously with about 10^4 viable TA3/Ha tumor cells were dissected 2 to 3 weeks post-injection at which time some metastatic lesions were macroscopically visible in the lungs and liver of the mice. These lesions were then dissected out of the respective organs and grown in culture for about a week before an aliquot of the selected tumor cells were reinjected intravenously into healthy strain A/J mice and the whole selection procedure repeated. In the process of selecting specific lung metastatic tumor variants, it was found that the first passage of the tumor cells *in vivo* resulted in about 10 lung and 3 liver tumor lesions per animal. The second passage saw fewer liver lesions but about the same number of lung lesions per mouse. Passage 3 through 8 resulted in very few visible liver lesions but an overabundance of lung lesions.

In the selection of liver specific metastatic tumor variants, the first passage of the cells *in vivo* resulted in about 16 liver lesions and between 100-200 lung lesions per mouse. The second animal passage gave more than 50 liver lesions and very few lung foci. It was found that from the fifth *in vivo* passage on, no lung nodules were visible while the liver had extensive metastatic lesions. Based on the metastatic characteristics of the specific metastatic lung and liver tumor variants, tumor cells that were cultured from the animal passage number 5 for the TA3/Ha liver specific metastatic tumor variants and passage number 8 for the lung specific metastatic variants were used for the *in vivo* biodistribution studies.

Biodistribution studies

The availability of the TA3/Ha liver and lung specific metastatic tumor variants allows the study, by scintigraphic imaging and biodistribution studies, of the *in vivo*

avidity of the peanut lectin for these metastatic tumors based on the known high PNA avidity of the solid tumor form from which these metastatic tumor variants arose. A correlation of the metastatic potential of the TA3/Ha tumor cells has been found with the tumor cells' surface epiglycanin content[235], of which 60% of the surface oligosaccharide chains present are T-antigenic in structure[246]. Radioiodinated peanut lectin was used here to determine whether the selected lung and liver metastatic tumor variants still retained their cell surface T-antigenic epiglycanin structures, and also to examine the potential utility of PNA as a radioimaging agent in the detection of lung and liver metastatic tumor variants *in vivo*. The results of the *in vitro* cell binding study (table 2) performed with the cells of the TA3/Ha metastatic lung and liver variant tumors and radioiodinated peanut lectin have shown that these cells do possess numerous PNA receptors. It appears that the TA3/Ha metastatic tumor cells did not lose their surface T-antigenic structures in the selection process.

Tables 11 and 12 give the results of the biodistribution study performed on mice bearing the TA3/Ha specific lung metastatic variant tumor with ^{125}I-PNA as the radiolocalizing agent. The mice used had been injected with the metastatic lung specific tumor cells and the tumor was allowed to grow in the animals for fifteen days. These mice were then injected with the labeled peanut lectin and 8 hours post-injection, the mice were sacrificed and the various organs of interest dissected out. A group of normal, healthy mice were used as controls. These control mice were of the same age as the tumor bearing mice and were injected with normal saline instead of the tumor cells. Gross anatomical examination of the various organs showed that the tumor infiltrated lungs from the TA3/Ha metastatic lung variant tumor bearing animals were about twice the mass of the controls. These lungs were also found to contain multiple tumor lesions. Varying degrees of splenomegaly was also observed in the tumor bearing mice. The mean whole body weight of the tumor bearing mice was found to be 86 ± 8.2% that of the same age controls. The concentration of ^{125}I-PNA found circulating in the

Table 11: Biodistribution of ^{125}I-PNA in A/J mice bearing TA3/Ha lung metastatic variant tumors[1,2]

Tissues	Control mice	Tumor bearing mice
Blood	2.21 (1.41-3.04)	5.53* (4.40-6.24)
Salivary glands	13.75 (7.67-20.31)	23.97(NS) (10.75-31.66)
Spleen	1.53 (1.06-2.30)	5.67** (3.57-7.11)
Stomach†	13.27 (8.60-20.51)	12.95(NS) (8.81-19.62)
Kidneys	13.63 (10.76-19.24)	51.19* (16.39-77.08)
Muscle	(0.33-[illegible])	1.28* (0.96-1.77)
Lungs	3.20 (2.48-4.23)	12.33** (8.54-19.25)
Liver	1.36 (0.96-1.81)	3.54* (2.65-5.71)

[1]Values expressed as the mean percent injected dose per gram of tissue 8 hours post-injection of ^{125}I-PNA.

[2]The values in the parentheses give the range obtained from the 5 mice in each group.

*Statistically significant from controls $p < 0.05$ (Student paired t-test)

**Statistically significant from controls $p < 0.01$

NS = not significantly different from controls

† including contents

Table 12: Uptake of ^{125}I-PNA by whole organs of A/J mice bearing TA3/Ha lung metastatic variant tumors[1,2]

Tissues	Control mice	Tumor bearing mice
Salivary glands	2.62	3.95(NS)
	(1.36-4.21)	(2.98-6.77)
Spleen	0.12	0.45**
	(0.09-0.16)	(0.23-0.75)
Stomach†	4.93	4.08(NS)
	(2.46-7.58)	(2.08-8.42)
Kidneys	4.98	12.58*
	(3.90-6.96)	(6.01-15.88)
Lungs	0.48	3.87**
	(0.39-0.58)	(2.27-8.07)
Liver	1.77	3.10*
	(1.38-2.33)	(2.06-5.10)

[1]Values expressed as the mean percent injected dose per whole organ 8 hours post-injection of ^{125}I-PNA.

[2]The values in the parentheses give the range obtained from the 5 mice in each group.

*Statistically significant from controls $p < 0.05$ (Student paired t-test).

**Statistically significant from controls $p < 0.01$

NS = not significantly different from controls

† including contents

blood of the tumor bearing mice was calculated to be about 2.5 times that of the controls. This high level of radioactivity in the blood of the tumor bearing mice most probably contributed to the high levels of radioactivity accumulation in the other organs (tables 11 and 12). Uptake of radioiodinated peanut lectin was found to be about 3.8 times as much in the tumor infiltrated lungs compared to the controls on a per gram basis. On the whole organ level, the lungs from the tumor bearing mice were calculated to have 8 times as much radioactivity as the controls. The TA3/Ha tumor infiltrated lungs had the most significant uptake of radiolabeled peanut lectin among all of the various organs investigated in comparison to the organs from the control animals. The spleens of these tumor bearing mice had, on the average, an uptake of ^{125}I-PNA that was 3.7 times that of the controls when compared at both the per gram basis as well as the whole organ. There were some macroscopically obvious tumor nodules seen in some of the livers of the tumor bearing mice and these nodules were especially visible in the animals that had extensive lung metastatic lesions. The accumulation of ^{125}I-PNA in the liver of the tumor bearing mice was found to be, on the average, 2.6 times that of the controls in comparison at the per gram of tissue basis and about about 1.8 times at the whole organ basis. This increase in uptake of PNA by the liver of the tumor bearing mice could be due to the binding of the peanut lectin to micrometastatic liver lesions. It is also possible that epiglycanin shed from the TA3/Ha tumor in the lung formed a complex with the peanut lectin and was being filtered from the circulation by the liver, thus contributing to the increased radioactivity accumulation. The amount of radioactivity found in the kidneys of the tumor bearing mice was found to vary greatly but the uptake was found to be significantly higher than that of the control animals although no visible abnormalities or change in weight and size of the kidneys were evident. The other organs examined as given in tables 11 and 12 were calculated to be not statistically significantly different from those of the tumor bearing mice and the controls.

The results of a 24 hour biodistribution study with radiolabeled peanut lectin performed on A/J mice bearing the specific TA3/Ha liver variant metastatic tumor are presented in tables 13 and 14. These liver tumor bearing mice were found, upon dissection, to have enlarged spleens, livers and lungs. The liver in the tumor bearing mice were found covered with tumor nodules and in some of the livers, the number of tumor lesions were too numerous to count. Although enlargement of the various organs were found in the tumor bearing mice, the mean whole body weight of these animals was found to be 88 ± 5.6% that of the same age control animals. In comparing the accumulation of radioactivity in the various organs between the control animals and the TA3/Ha liver specific metastatic variant tumor bearing mice, it was found that the blood, liver and spleen were the organs that had statistically significant differences in radioiodinated peanut lectin uptake. ^{125}I-PNA concentrations in the blood circulation is usually low at 24 hours post-injection of the radioiodinated peanut lectin as seen in previous studies. This was not the case in the metastatic liver variant tumor bearing mice which were found to have blood concentrations levels of ^{125}I-PNA twice that found in the blood circulation of the control mice. The splenic mass in the tumor bearing mice was found to be about twice as much as the spleen in the control animals. The uptake of radioactivity, however, in the enlarged spleens was found to be 4.8 times as much as the controls when compared on the per gram of tissue basis. The entire spleen of the tumor bearing mice, on the average, accumulated 6.2 times that of the normal spleen in the control animals. The lungs in the metastatic liver variant tumor bearing mice were slightly enlarged and some occasional small lesions were seen. The uptake of radioiodinated peanut lectin in the lungs, however, was not found to be significantly different from that of the control animals. The tumor infiltrated liver was, on the average, 1.3 times the mass of the controls but these tumor bearing livers had a mean ^{125}I-PNA concentration that was about 5.8 times that of the controls on a per gram basis comparison. Some individual livers with extensive metastatic tumor

Table 13: Biodistribution of ^{125}I-PNA in A/J mice bearing TA3/Ha liver metastatic variant tumors[1,2]

Tissues	Control mice	Tumor bearing mice
Blood	0.63 (0.54-0.76)	1.24* (0.92-2.09)
Salivary glands	6.86 (3.18-9.37)	9.05(NS) (4.38-18.32)
Spleen	0.92 (0.65-1.04)	4.42* (2.06-9.18)
Stomach†	4.50 (1.43-8.10)	5.08(NS) (2.21-8.76)
Kidneys	13.24 (11.03-16.71)	16.56(NS) (10.25-26.47)
Muscle	0.28 (0.23-0.35)	0.72* (0.29-1.20)
Lungs	2.85 (2.44-3.57)	3.77(NS) (1.55-4.97)
Liver	1.15 (0.92-1.29)	6.76* (4.00-14.35)

[1]Values expressed as the mean percent injected dose per gram of tissue 24 hours post-injection of ^{125}I-PNA.

[2]The values in the parentheses give the range obtained from the 5 mice in each group.

*Statistically significant from controls $p < 0.05$ (Student paired t-test).

NS = not significantly different from controls

† including contents

Table 14: Uptake of ^{125}I-PNA by whole organs of A/J mice bearing TA3/Ha liver metastatic variant tumors[1,2]

Tissues	Control mice	Tumor bearing mice
Salivary glands	0.98	1.04(NS)
	(0.50-1.43)	(0.45-2.09)
Spleen	0.17	1.06*
	(0.15-0.18)	(0.42-2.31)
Stomach†	2.36	1.82(NS)
	(0.88-4.01)	(0.85-3.50)
Kidneys	3.77	4.57(NS)
	(2.13-5.20)	(3.11-6.73)
Lungs	0.42	0.73(NS)
	(0.36-0.54)	(0.25-1.24)
Liver	1.44	11.33*
	(1.35-1.69)	(5.36-14.35)

[1]Values expressed as the mean percent injected dose per whole organ 24 hours post-injection of ^{125}I-PNA.

[2]The values in the parentheses give the range obtained from the 5 mice in each group.

*Statistically significant from controls $p < 0.05$ (Student paired t-test).

NS = not significantly different from controls

† including contents

infiltrations were found to have ^{125}I-PNA concentrations that were as high as 14% of the injected dose per gram of tissue compared to the range of 0.9 ± 1.3% percent of the injected dose obtained for the normal liver in the control animals. The whole liver mass of the tumor bearing animals was found to have a mean accumulation of ^{125}I-PNA that was 7.8 times that of the normal liver. Even though the blood concentration of ^{125}I-PNA was about twice that of the controls, it could not fully account for the observed greater increased concentration of peanut lectin seen in the livers and spleens of the tumor bearing animals where radiotracer uptake was found to be statistically significantly different from the controls. By far, the uptake of PNA by the liver was found to have the greatest difference among the various organs studied in comparison to the control animals as seen in tables 13 and 14. Splenomegaly was quite obvious in the animals bearing the liver metastatic variant tumor but no visible lesions were found in the spleen of the mice. The reason for the observed increased uptake of ^{125}I-PNA by the spleen of the tumor bearing animals has not yet been elucidated. It is possible that the presence of the tumor in the animal may cause a proliferation of splenic macrophages and experiments performed have found that the splenic macrophages do bind the peanut lectin *in vitro*. High levels of ^{125}I-PNA uptake were also seen in the salivary glands, stomach and kidneys of both sets of mice, tumor bearing and control. The accumulation of radioactivity in the salivary glands, stomach and kidneys in the control and tumor bearing mice were not found to be statistically different (tables 13 and 14).

From the two sets of biodistribution studies performed on the selected metastatic lung and liver tumor variants, the results indicate that the radioiodinated peanut lectin does localize in the organs that bear the metastatic tumors. Tissue to blood ratios calculated for the mice bearing the lung and liver metastatic tumor variants indicate that a good delineation of the tumor infiltrated organ could be obtained by the process of scintigraphic imaging using radiolabeled peanut lectin as the tumor localizing

agent. As seen in the results, normal lungs and liver do not take up as much of the ^{125}I-PNA as the tumor bearing organs. Hence, intense localization of the radiolabeled peanut lectin in the lungs and liver would probably indicate the presence of exposed T-antigenic binding sites, a condition not normally seen under healthy conditions.

Whole body scintigraphy

The results obtained from the biodistribution studies with the metastatic tumor variant bearing mice indicate the potential utility of the radioiodinated peanut lectin as a radioimaging agent in the detection of T antigen metastatic tumors. Whole body gamma scintigraphic imaging was then performed on mice bearing the specific TA3/Ha metastatic lung and liver tumor variants to determine whether the results obtained from the biodistribution studies can be extrapolated to external non-invasive imaging techniques. The whole body gamma scintigraphic images obtained from the tumor bearing mice are presented as figures 17 and 18. Although the biodistribution data indicated that some background activity would be present in the whole body, the scans obtained gave good delineation of the tumor bearing organs. Figure 19 is a whole body scintigraphic image of a mouse bearing a solid subcutaneous TA3/Ha tumor taken 24 hour post-injection of ^{125}I-PNA and is used here for comparison. The whole body scan of the solid tumor bearing mouse shown in figure 19 illustrates increased areas of ^{125}I-PNA uptake in the kidneys, tumor and some in the stomach. Only a small amount of background activity is seen in the lungs and liver. Figure 17 represents the whole body image taken of a mouse bearing the TA3/Ha metastatic lung tumor variant 10 hours post-injection of ^{125}I-PNA. The scan here [illegible] good delineation of the lungs even though high body background activity is s[illegible]tion of the same animal, the lungs were found to contain multiple large t[illegible]ure 18 is a representative whole body scan of a mouse bearing the specific TA3/Ha liver metastatic tumor variant 24 hours post-injection of the radiolabeled peanut lectin. This scan clearly shows the liver as a region of concentrated radioactivity besides the usual areas of radioactivity in the kidneys and stomach and

Figure 17 Whole body gamma scintigraphic image of an A/J mouse bearing the TA3/Ha lung metastatic tumor variant 10 hours post-injection of ^{125}I-PNA (2MBq;4μg PNA)

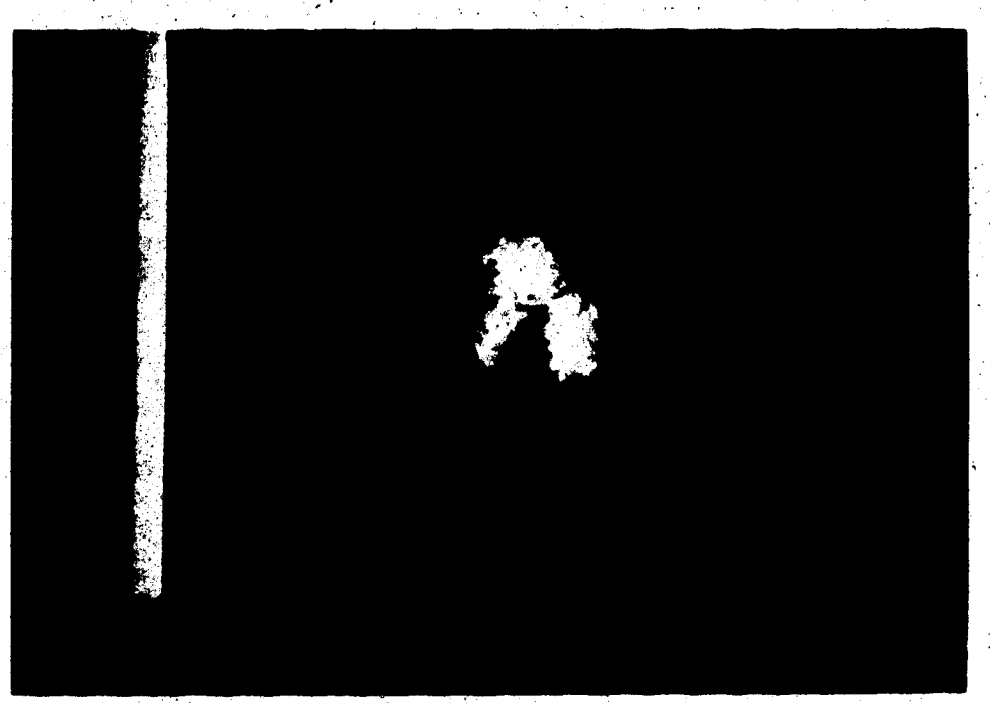


Figure 18 Whole body gamma scintigraphic image of an A/J mouse bearing the TA3/Ha liver metastatic liver tumor variant 24 hours post-injection of ^{125}I-PNA (2MBq;4μg PNA)

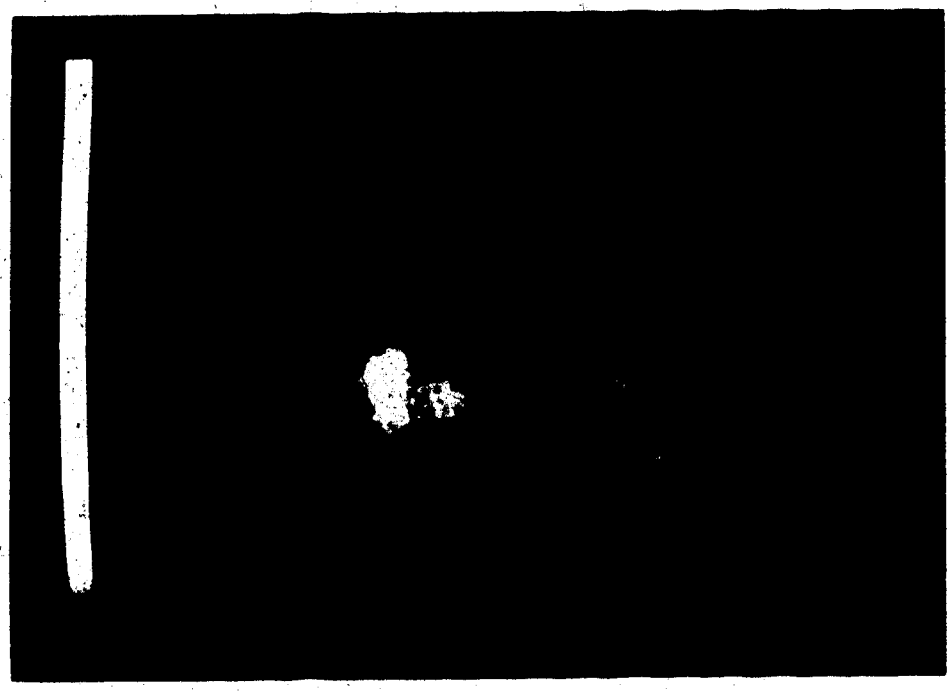


Figure 19 Whole body gamma scintigraphic image of an A/J mouse bearing a subcutaneous TA3/Ha solid tumor 24 hours post-injection of ^{125}I-PNA (2MBq;4μg PNA)

occasionally in the urinary bladder. When sacrificed and dissected for gross tissue examination, this liver tumor bearing mouse was found to have large and many tumor nodules throughout the liver. It was further noted that some mice that had been injected with the specific liver metastatic tumor variant cells did not produce a really positive scan upon the whole body scintigraphic imaging with radiolabeled peanut lectin. These mice were found, upon dissection, to have only a few small (1-2 mm) tumor lesions in the liver. However, when the livers from these mice were dissected and the organ placed directly under the camera, an increased amount of radioactivity was seen at the areas of the tumor lesions. It is probably the limiting resolution of the gamma camera as well as dealing with small animals such as mice that small and few tumor lesions could not be detected well enough by the camera to produce a positive scan. In comparing the two scans of the animals bearing the specific TA3/Ha metastatic lung and liver tumor variants with the mouse bearing the TA3/Ha solid tumor (figures 17, 18 and 19), radioiodinated peanut lectin appears to be a useful radiolocalizing and radioimaging agent in these metastatic tumor models. In normal cases, the peanut lectin has been found not to localize in significant amounts in any of the organs except in the kidneys, stomach and occasionally in the bladder. In tumor bearing mice, however, as evidenced by the whole body images presented in figures 17, 18 and 19, and from the data obtained from the various biodistribution data, there is significant PNA uptake by the solid tumor or by the organs that are infiltrated with metastatic tumor lesions. Other organs not infiltrated with tumor lesions in the tumor bearing mice do not exhibit increased PNA uptake in comparison to the same organs in normal mice. Thus, PNA localization is evident in the TA3/Ha adenocarcinoma and its selected tumor variants *in vivo* and the radiolabeled peanut lectin is concentrated enough by the tumor to permit a non-invasive external imaging of the tumor site. The radiolabeled peanut lectin, therefore, does lend itself as a potential clinical radioimaging agent of T-antigen bearing tumors and their possible metastatic lesions.

Autoradiography and FITC-PNA staining

The presence of the Thomsen-Friedenreich antigen can be detected in tissue sections with the use of labeled PNA by a variety of immunohistological methods. Autoradiography with ^{125}I-PNA and staining with FITC-PNA are two such methods in detecting the T-antigen in tissue sections. Figure 20 shows the gross autoradiography of a cryostat tissue section taken from a liver lobe that was found to be infiltrated with tumor nodules in a mouse bearing the specific TA3/Ha metastatic tumor variant. Figure 21 is the tissue section of a liver lobe infiltrated with specific TA3/Ha metastatic tumor variant lesions that is stained with hematoxylin and eosin. This same mouse had been injected with ^{125}I-PNA (2 MBq;4 μg PNA) 24 hours before the removal of the tumor infiltrated liver. The autoradiograph presented shows intense localization of the peanut lectin throughout the viable tumor masses. A higher intensity of PNA localization is seen especially in the smaller dense metastatic nodules as well as in the outer edges of the larger tumor masses. Micrometastatic lesions in the tumor bearing liver could be identified in the autoradiograms of the tissue sections that had been superimposed onto histological processed tissue sections. These micrometastatic lesions were found to appear as small and dark pinpoints that had darkened the autoradiographic film with the localization of ^{125}I-PNA. Although the technique of autoradiography with ^{125}I-PNA does not allow a high enough resolution to exactly determine the level of PNA binding at the cellular level, the gross autoradiogram presented in figure 20 does give a clear indication of the avid PNA binding by the metastatic tumor masses in the tissue section of the liver. This is depicted by the areas of increased intensity of radioactivity. It was also further observed that the smaller metastatic tumor lesions in the liver had a uniform distribution of intense radioactivity hence, implying that these smaller lesions expressed the T-antigen throughout the tumor mass and that the intravenously administered ^{125}I-PNA was readily accessible to all areas of the tumor. In the larger tumor masses that had necrotic areas the binding of the peanut lectin was limited mainly to the viable areas of the tumor tissue. The autoradiograph of a cryostat tissue section taken from the liver lobe of a mouse bearing the specific TA3/Ha metastatic liver variant tumor

Figure 20 Autoradiogram of a liver tissue section taken from an A/J mouse bearing the TA3/Ha liver metastatic tumor variant 24 hours post-injection of I-125 PNA (X25).

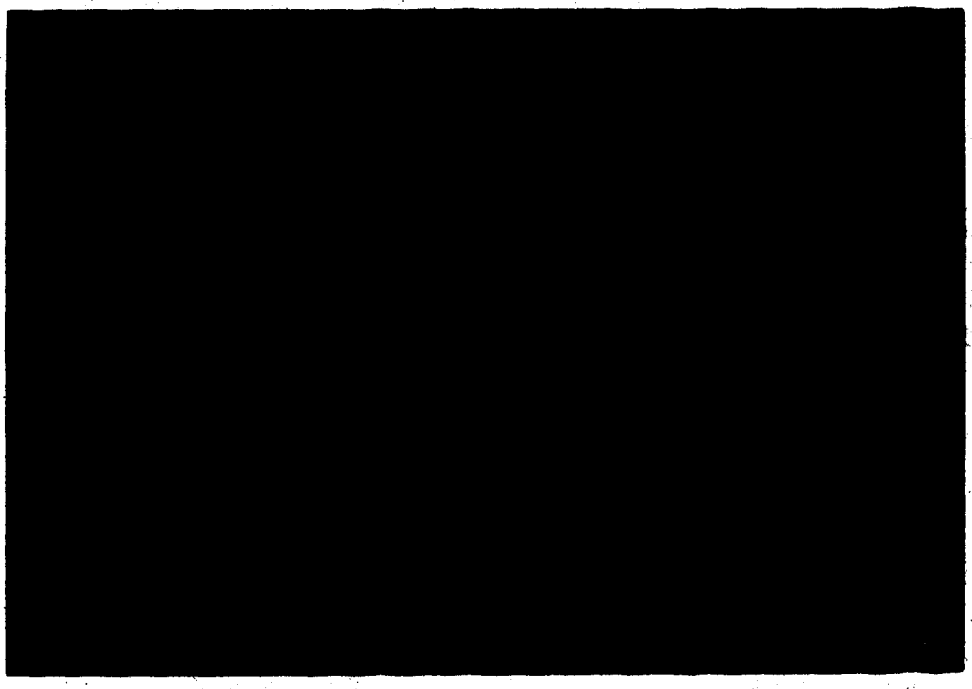


Figure 21 Hematoxylin and eosin stain of a liver tissue section taken from an A/J mouse bearing the TA3/Ha liver metastatic tumor tumor (X250).

which had been injected with ^{125}I-PNA five days before is shown in figure 22. A clearly different pattern of PNA localization is seen here in comparison to the autoradiograph of the tissue section taken 24 hours post-injection of the peanut lectin. This autoradiogram, figure 22, reveals a highly intensive accumulation of the ^{125}I-PNA in the central necrotic areas of the tumor masses while very little localization was found in the outer viable areas of the rapidly growing tumor mass. This observation clearly implies that PNA is indeed retained by the tumor mass. This retention of ^{125}I-PNA five days after injection in the area of necrosis in the tumor mass is probably due to the binding of PNA to epiglycanin and forming a complex. This PNA-epiglycanin complex was probably shed from the surface of the viable tumor cells into the necrotic area, thus entrapping the complex in the central necrotic area of the tumor mass which is lacking of a vascular supply. The outer viable areas of the tumor mass is seen to be rather "cleared" of ^{125}I-PNA localization (figure 22) after 5 days. This is most probably due to the tumor cells shedding off their surface epiglycanin and the PNA bound to these molecules would also be shed along from the tumor surface. This is consistent with reports that the TA3/Ha tumor line has a high turnover rate of epiglycanin from the tumor cell surface[232,247]. It has been found that the overall release of labeled epiglycanin from the cell surface of the TA3/Ha tumor followed a biphasic kinetics in which 20% of the label was shed with a half-life of 45 minutes and the remaining 80% with a half-life of 40 hours[232]. The autoradiograms presented, figures 20 and 22, support the the concept of PNA binding to cell surface epiglycanin and the rapid rate of epiglycanin turnover. This data, when extrapolated to radioimmunoimaging procedures, indicate that it is necessary to determine the turnover rate or amount of antigen shedding from a tumor cell surface prior to establishing radioimaging procedure as a delayed scan may result in low concentrations of the radiolocalizing agent at the tumor site due to shedding of the localizing agent-antigen complex.

An immunohistological technique that provides a high resolution of the binding activity in tissue sections is the use of FITC-PNA to stain the tissue sections. Identification of T-antigen expressing tumor lesions have been performed on various human tissue sections with

Figure 22 Autoradiogram of a liver tissue section taken from an A/J mouse bearing the TA3/Ha liver metastatic tumor variant 5 days post-injection of ^{125}I-PNA (X40).

the fluorochrome PNA tissue staining technique such as in human breast tissue[94,129], colon[134], kidneys[134], and at rectosigmoid sites[134]. Figure 23 illustrates the fluorescence microscopy photograph of a tissue section of tumor infiltrated liver lobe taken from a mouse bearing the TA3/Ha liver metastatic tumor variant. Binding of the peanut lectin can be seen to occur throughout the tumor mass. The outer viable proliferating areas of the tumor mass found to bind PNA was, under higher microscopic magnification, observed to bind the peanut lectin at the cell membrane. Individual micrometastatic lesions have also been found to bind the FITC-PNA and these lesions can be readily identified from their strong emission of fluoresence. The identification of micrometastatic lesions with such avid binding with PNA may help explain the observation in biodistribution studies where increased levels of ^{125}I-PNA localization was observed in the lungs and liver of the metastatic tumor bearing mice that were macroscopically devoid of any tumor lesions.

From the results obtained with the two immunohistological techniques, FITC-PNA staining and autoradiograms of the tissue sections, it can be seen that PNA binding sites are available throughout the viable TA3/Ha tumor mass. The autoradiograms demonstrate that the intravenous injected radioiodinated peanut lectin is able to penetrate throughout the tumor structure. The permeability of the TA3/Ha metastatic tumor vasculature is such that the radiotracer is distributed uniformly and rapidly throughout the tumor mass. The high turnover rate of epiglycanin is reflected by the difference in the autoradiograms obtained from the mice that were sacrificed 24 hours post-injection and 5 days post-injection of ^{125}I-PNA. The localization of the peanut lectin within the central necrotic areas of the tumor mass seen 5 days post-injection of the ^{125}I-PNA is most probably due to the PNA complexing with the epiglycanin of the tumor cell surface and subsequent shedding into the central necrotic area of the tumor mass while rapid growth continued on the outside of the tumor. The FITC-PNA staining of the tumor showed a positive staining even in the central and possibly necrotic areas of the tumor. The central necrotic areas that bind the FITC-PNA is probably due to the presence of shed epiglycanin entrapped within the necrotic space.

Figure 23 Fluorescence microscopy photograph of a liver tissue section of an A/J mouse, bearing the TA3/Ha metastatic liver tumor variant stained with FITC-PNA (X250).

The various studies, including the *in vitro* PNA binding with the selected TA3/Ha lung and liver metastatic tumor variant cells, biodistribution studies, histochemical studies with FITC-PNA staining, autoradiograms and the scintigraphic imaging of the selected TA3/Ha metastatic lung and liver tumor variants, illustrate that the selected TA3/Ha metastatic tumors do retain the binding affinity and tumor cell surface antigen expression of the parent TA3/Ha adenocarcinoma tumor cell line. This tumor cell line and its metastatic variants provides a useful animal tumor model to evaluate radiopharmaceuticals in the field of diagnostic oncology as it allows the determination of whether the probe in question retains the same avidity for the metastatic variants as the primary tumor. The TA3/Ha tumor cell line is also of great research interest due to the similarity of the surface immunodominant structures to those that are found on some human tumors[39,50,58] such as the Thomsen-Friedenreich antigen. The T antigen has been referred to as a human tumor associated antigen and has also been proposed as one of the factors involved in the invasion and metastatic spread of cancer[36]. Furthermore, the TA3/Ha animal tumor model presents the properties of antigen masking by the presence of endogenous glycoproteins which have been found to be related to the allotransplantability of this tumor line and this property has also been found to be possibly related also to the metastatic spread of human cancers[212]. The radiolabeled peanut lectin has demonstrated avid binding for the selected TA3/Ha metastatic lung and liver tumor variants both *in vitro* and *in vivo* as seen from the results of the various studies done. Radioiodinated peanut lectin has been established as a potential radioimaging agent based on the data provided by the TA3/Ha murine mammary adenocarcinoma animal model. There was sufficient concentration of the peanut lectin at the tumor site with minimal background activity to perform scintigraphic imaging without the need of any data manipulation to obtain clear delineation of solid subcutaneous tumors as well as metastatic lesions.

C. Anti-epiglycanin, murine IgM monoclonal antibody

Radioimmunodiagnosis of tumors by means of radiolabeled antibodies was first reported in 1957[17,137]. However, the use of antisera has always been burdened with numerous problems such as heterogeneity of antibodies, cross-reactivity and non-specific binding[146,147]. It has been hoped that with the development of the hybridoma technique[9], monoclonal antibodies would alleviate such problems faced with the use of antisera. Monoclonal antibodies recognize only one determinant and are of one class being derived from a single cell[146,147]. The use of appropriately labeled monoclonal antibodies would thus be of potential use in the detection and possibly therapy of cancers if monoclonal antibodies could be obtained that are specific for tumor associated antigens only.

Anti-epiglycanin is a murine monoclonal antibody that is active against the epiglycanin structures that are present at the surface and shed by the TA3/Ha tumor cells. The anti-epiglycanin monoclonal antibody is of experimental interest as it is specific for certain antigens present on the TA3/Ha murine adenocarcinoma cells, and this well studied tumor line has been found to bear some similarities to the human adenocarcinoma in some of its surface antigens[39,50]. It is thus hoped that anti-epiglycanin would yield additional data on the application of techniques involving the use of radiolabeled monoclonal antibodies for radioimmunodiagnosis.

In vitro studies with anti-epiglycanin

Radioiodination

Affinity purified anti-epiglycanin IgM monoclonal antibody was obtained from Dr. B.M. Longenecker, Department of Immunology, University of Alberta as a sterile solution in concentrations of 0.7 to 1.0 mg. of protein per mL of PBS. Anti-epiglycanin was routinely labeled with ^{125}I using the iodogen method[267,268] with labeling efficiencies of up to 65% to yield specific activities of approximately 150 kBq/μg of protein for animal biodistribution studies and about 700 kBq/μg for

radioimmunoimaging studies. Precipitation of an aliquot of the final radiolabeled product with 25% TCA usually resulted in greater than 98% of the total radioactivity being precipitated. The final radioiodinated anti-epiglycanin IgM product was always subjected to ultracentrifugation before use. It was found that centrifuging the solution of radiolabeled anti-epiglycanin at 100 000 x G for 24 hours resulted in about 80% of the total radioactivity settling at the bottom of the tube, most probably due to the presence of some protein aggregation. The formation of microaggregates is most likely due to the process of the radioiodination[253]. Hence, all the IgM monoclonal antibodies used were subject to ultracentrifugation before use and only the supernatant of the centrifuged solutions were used. Quantitation of the proteins in the supernatant of the solution was performed using the Bio-Rad protein assay kit. The uv absorbance curve generated with the standard supplied by the manufacturer was found to be similar to a curve generated with the use of an IgM solution standard made. Hence, all further protein quantitation of the IgM antibodies was compared to that of the manufacturer's supplied standard.

Trichloroacetic acid precipitation of the radiolabeled anti-epiglycanin monoclonal antibody showed radiochemical purity greater than 95% after three days and greater than 90% after seven days following radioiodination when stored at 4° C and at a pH of 7.4.

Cell-binding studies

To examine the activity of anti-epiglycanin against the T-antigen, radioiodinated anti-epiglycanin was incubated with various human blood group type erythrocytes that had been treated with neuraminidase to expose the T-antigen. It was found that upon incubation of the neuraminidase treated red blood cells with anti-epiglycanin, hemagglutination of the cells occured whereas this phenomena was not observed with normal red blood cells. The results of the blood cell binding assay of anti-epiglycanin with the various human erythrocytes and TA3/Ha cells are given in

table 15. It was seen that the presence of D-galactose in the reaction mixture did very little to the anti-epiglycanin-N'RBC or anti-epiglycanin-TA3/Ha cell interactions at all. This phenomena is of considerable interest as the presence of D-galactose in the peanut lectin-N'RBC reaction mixture resulted in a strong inhibition of the PNA binding of exposed T-antigen on the N'RBC as seen in table 1. The binding of epiglycanin to N'RBC and to the TA3/Ha tumor cells was found to be virtually unaffected by the presence of galactose. A small amount of binding was seen with normal human red blood cells which could be due to some entrapment of the antibody between the cells. Hence, anti-epiglycanin is somewhat similar to the peanut lectin in that both agglutinate neuraminidase treated red blood cells and have high affinity for the TA3/Ha adenocarcinoma tumor cells but have an insignificant amount of binding to normal red blood cells. The difference found in the binding activity of the two different proteins is that the peanut lectin's binding activity could be inhibited by the presence of galactose whereas the activity of anti-epiglycanin was found to be unaffected by the presence of that sugar. This then implies that the peanut lectin and anti-epiglycanin may have affinities for the same cells but have different binding sites. To further investigate this difference in binding of the two proteins for the same TA3/Ha tumor cells, TA3/Ha tumor cells were incubated with a mixture of PNA and anti-epiglycanin. This study showed that both proteins bound to the tumor cells in the same proportions as if the other protein was not in the mixture. Hence, the presence of the peanut lectin in the reaction mixture of anti-epiglycanin and TA3/Ha tumor cells did not have any effect on the binding of the monoclonal antibody and vice versa as seen in table 16. This further substantiates the finding that the peanut lectin and anti-epiglycanin monoclonal antibody have different binding sites on the same cell. This observation stimulated interest in determining the effect of the simultaneous administration of PNA and anti-epiglycanin in the TA3/Ha animal tumor model as the monoclonal antibody may be able to enhance the scintigraphic image obtained with

Table 15: Cell binding studies with ^{125}I-anti-epiglycanin[1,2].

	Blood group type			
	A	B	AB	O
N'treated RBC[3]	22.56±3.57	24.40±1.77	24.33±2.79	24.43±1.16
+ PNA(0.5μg)	19.45±2.31	19.08±2.67	24.36±1.12	22.09±1.02
+ 0.1M galactose	19.85±0.15	18.43±1.10	22.95±2.75	19.54±1.35
Normal RBC[4]	11.94±2.53	10.52±1.12	11.37±1.41	12.30±1.62
+ PNA(0.5μg)	10.50±1.08	10.05±1.01	10.70±0.96	11.93±0.05
TA3/Ha cells[5]	14.31±0.37			
+ PNA(0.5μg)	14.05±0.03			
+ 0.1M galactose	13.70±1.20			

[1]0.5 μg of anti-epiglycanin was used in each determination.

[2]$n = 5$

[3]approximately 0.75 x 10^7 cells used.

[4]approximately 1.20 x 10^7 cells used.

[5]approximately 5.00 x 10^5 cells used.

Table 16: Binding of the peanut lectin and anti-epiglycanin to TA3/Ha tumor cells[1].

Amount of PNA added[2]	%binding of anti-epiglycanin to TA3/Ha cells[3]	Amount of anti-epiglycanin added[4]	%binding of PNA to TA3/Ha cells[3]
0.0 μg	13.32±1.85	0.0 μg	34.10±1.31
5.0	13.70±1.20	5.0	38.6±1.53
50.0	13.05±0.31	12.5	36.51±1.53
120.0	11.78±0.92	25.0	33.72±1.37
250.0	12.78±0.40	50.0	34.12±0.33
500.0	10.07±0.95		

[1] $n = 5$

[2] The amount of anti-epiglycanin in each determination was 0.5 μg with increasing amounts (μg) of PNA added.

[3] approximately 5.0 x 10^5 viable TA3/Ha tumor cells used in each determination.

[4] The amount of PNA in each determination was 0.5 μg with increasing amounts (μg) of anti-epiglycanin antibodies added.

radiolabeled PNA or vice-versa. Also, the *in vitro* cell binding results of anti-epiglycanin appear promising enough to further investigate the potential of this monoclonal antibody as a possible radioimmunoimaging agent.

In vivo studies with anti-epiglycanin IgM monoclonal antibodies

Preliminary studies on the *in vivo* biodistribution of radioiodinated anti-epiglycanin monoclonal antibody were performed on A/J mice bearing subcutaneous TA3/Ha adenocarcinoma. Another IgM monoclonal antibody 49H.24 which has been reported not to have any binding affinity for the TA3/Ha tumor cells[274] *in vitro* was used as the non-specific tissue localization control. The results of the study are presented in table 17 as the percent uptake of ^{125}I-anti-epiglycanin per gram of tissue and in table 18 as the percent uptake of the injected anti-epiglycanin dose per organ at the different time periods studied. The data obtained indicate a high whole body retention of the anti-epiglycanin monoclonal IgM antibody at 6 hours post-injection with most of the radioactivity in the circulation. The anti-epiglycanin does not seem to be retained in the whole body for prolonged periods as the percent of the total dose injected that is found remaining in the whole body dropped to 12.7% at 24 hours and a low 3.5% at 48 hours post-injection. Most of the radioactivity at all the time periods studied was found to be in the blood circulation as expected of a molecule with such a high molecular weight (900 000 daltons). The non-specific control 49H.24 monoclonal antibody was found to behave in a somewhat similar manner to that of anti-epiglycanin in the biodistribution of the IgM in the various tissues but 49H.24 was retained longer in the body. Most of the 49H.24 activity was seen in the blood with a fair amount of radioactivity also seen in the liver. Blood plasma samples obtained from the mice and analyzed for the amount of free radioiodide in circulation due to the breakdown of the label *in vivo* revealed that there was only about 10-20% of the total radioactivity in circulation due to free radioiodide at all the time periods studied for both the anti-epiglycanin and the non-specific 49H.24 control. Thus, the radioactivity seen in most of the tissues would have little contribution from the free radioiodide. Calculating tissue

Table 17: Biodistribution of ^{131}I anti-epiglycanin and ^{131}I 49H.24 monoclonal antibodies in A/J mice[1] bearing subcutaneous TA3/Ha solid tumors[2].

	Anti-epiglycanin monoclonal antibody				49H.24 monoclonal antibody			
Tissues	6 hours	24 hours	48 hours	72 hours	6 hours	24 hours	48 hours	72 hours
Blood	10.26±3.30	1.45±0.38	0.29±0.10	0.13±0.02	17.27±4.49	3.76±0.28	0.92±0.29	0.51±0.11
Salivary glands	20.12±5.81	2.71±1.13	0.29±0.07	0.20±0.03	23.01±9.81	4.31±1.72	0.66±0.15	0.41±0.07
Spleen	6.53±2.18	1.13±0.82	0.19±0.07	0.14±0.06	4.69±0.89	1.33±0.44	0.42±0.06	0.28±0.05
Stomach	18.88±7.57	2.71±1.12	0.27±0.05	0.21±0.03	23.64±9.18	4.21±1.78	0.54±0.13	0.39±0.08
GIT	2.75±1.04	0.43±0.15	0.07±0.01	0.05±0.01	4.25±1.72	0.80±0.29	0.18±0.04	0.14±0.03
Kidneys	5.44±1.41	0.97±0.31	0.30±0.05	0.22±0.05	9.72±1.76	2.96±0.87	1.42±0.24	0.99±0.16
Muscle	0.95±0.31	0.17±0.06	0.03±0.01	0.03±0.01	1.31±0.39	0.35±0.14	0.13±0.03	0.10±0.02
Lungs	5.81±1.74	0.83±0.24	0.16±0.03	0.09±0.01	9.03±3.06	1.88±0.53	0.50±0.11	0.28±0.06
Liver	6.16±1.43	0.79±0.29	0.16±0.03	0.11±0.01	8.25±1.58	2.03±0.52	0.93±0.16	0.69±0.07
TA3/Ha tumor	7.23±2.49	2.83±1.32	1.20±0.67	0.81±0.17	8.69±2.72	4.03±1.70	1.64±0.76	1.01±0.51

[1] n = 8

[2] Values are expressed as mean percent dose per gram of tissue ± standard deviation.

Table 18: Uptake of ^{131}I anti-epiglycanin and ^{131}I 4911.24 monoclonal antibodies in various organs of A/J mice[1] bearing subcutaneous TA3/Ha solid tumors[2].

	Anti-epiglycanin monoclonal antibody				4911.24 monoclonal antibody			
Tissues	6 hours	24 hours	48 hours	72 hours	6 hours	24 hours	48 hours	72 hours
Blood	13.88±4.79	1.80±0.42	0.38±0.13	0.17±0.03	23.28±6.82	4.64±0.70	1.19±0.41	0.64±0.15
Salivary glands	3.47±1.32	0.39±0.14	0.05±0.02	0.03±0.00	4.03±2.15	0.61±0.21	0.10±0.04	0.06±0.01
Spleen	1.13±0.54	0.17±0.12	0.05±0.02	0.04±0.02	0.82±0.34	0.20±0.03	0.10±0.01	0.08±0.01
Stomach	4.88±1.79	0.86±0.39	0.09±0.03	0.05±0.01	6.09±2.09	1.33±0.62	0.17±0.04	0.09±0.02
GIT	5.65±1.75	0.81±0.24	0.14±0.03	0.08±0.01	8.70±2.91	1.50±0.35	0.36±0.06	0.25±0.03
Kidneys	1.90±0.48	0.30±0.06	0.09±0.01	0.07±0.01	3.39±0.60	0.92±0.14	0.44±0.05	0.31±0.02
Lungs	1.02±0.25	0.11±0.04	0.03±0.01	0.02±0.01	1.58±0.37	0.25±0.07	0.08±0.02	0.05±0.01
Liver	6.79±1.64	0.93±0.26	0.20±0.02	0.13±0.01	9.07±1.73	2.41±0.42	1.18±0.13	0.78±0.04
TA3/Ha tumor	1.82±0.74	1.08±0.46	0.60±0.16	0.38±0.23	2.21±0.22	1.58±0.28	0.88±0.42	0.55±0.32

[1] $n = 8$

[2] Values are expressed as mean percent dose per whole organ ± standard deviation.

to blood ratios for anti-epiglycanin, table 19, found that at 6 hours post-injection only the salivary glands and stomach had a higher ratios than 1.0. At 24 hours post-injection, the tumor was the tissue found to have the highest tissue to blood ratio. The tumor to blood ratio calculated for 49H.24 was also found to be the highest at 24 hours post-injection but the ratio obtained was not as high as that calculated for anti-epiglycanin. This could be due to the high concentration of 49H.24 levels in the blood.

Previous workers have addressed some of the problems associated with using high molecular weight proteins as tracers for tumor detection. The plasma half life of an IgM antibody in a mouse after i.v administration has been found to be between 0.2 to 0.6 days and an IgM molecule with its large molecular weight of approximately 900 000 daltons tends to remain largely in the circulation and not diffuse out into the extravascular space[279]. The blood supply of transplanted tumors have been found to be established by the formation of new capillaries from the surrounding tissue's blood vessels in the host[271]. These tumor capillaries have been found to be highly permeable and would allow almost unlimited passage of materials including blood erythrocytes[271] and also IgM molecules[280] into the extravascular space of the tumor. It was further reported that in some animal tumors, that high molecular weight molecules such as IgM antibodies had greater capillary permeability in tumor tissues than in most other normal organ tissues[210]. The extravascular space of the tumors have been found to be markedly larger than the those found in the normal tissues[116], and most large molecular weight proteins that diffuse into the extracellular space of the tumors are usually trapped[189,190]. Return of most of these trapped proteins to the intravascular space have been found to take place only by route of the lymphatic vessels[189]. Other factors have been reported to contribute to an artificial retention of high molecular weight radiotracers at the tumor site resulting in many of the false positives that have been cited[186,190].

The large molecular weight of the anti-epiglycanin and the non-specific 49H.24 probably accounts for the prolonged retention of radioactivity seen in the blood. It is possible that the highly vascularized TA3/Ha tumor with its probable increased tumor capillary wall

Table 19: Tissue to blood ratios of ^{131}I labeled anti-epiglycanin and ^{131}I 4911.24 monoclonal antibodies[1].

Tissues	Anti-epiglycanin monoclonal antibody				4911.24 monoclonal antibody			
	6 hours	24 hours	48 hours	72 hours	6 hours	24 hours	48 hours	72 hours
Salivary glands	2.01±0.42	1.82±0.60	1.08±0.42	1.54±0.33	1.44±0.80	1.12±0.35	0.76±0.2[illegible]	0.85±0.28
Spleen	0.68±0.24	0.78±0.25	0.79±0.58	1.00±0.68	0.28±0.08	0.35±0.05	0.50±0.21	[illegible]
Stomach	1.88±0.78	1.82±0.66	1.05±0.48	1.64±0.41	1.37±0.42	1.09±0.38	0.63±0.25	0.80±0.22
GIT	0.27±0.06	0.29±0.07	0.26±0.11	0.34±0.07	0.26±0.15	0.21±0.04	0.21±0.04	0.21±0.09
Kidneys	0.54±0.05	0.66±0.09	1.18±0.63	1.66±0.45	0.59±0.16	0.78±0.10	1.70±0.72	2.04±0.52
Muscle	0.09±0.02	0.12±0.02	0.11±0.04	0.24±0.10	0.08±0.03	0.09±0.02	0.15±0.06	0.20±0.04
Lungs	0.59±0.21	0.57±0.05	0.61±0.21	0.67±0.11	0.54±0.17	0.50±0.06	0.56±0.13	0.57±0.07
Liver	0.61±0.07	0.54±0.13	0.63±0.34	0.83±0.11	0.50±0.14	0.54±0.09	1.09±0.40	1.39±0.23
EA3/11a tumor	0.70±0.09	1.87±0.50	4.42±2.32	6.29±4.91	0.51±0.12	1.05±0.31	1.93±1.08	2.02±1.05

[1]n = 8

[2]Values are expressed as the mean ratio ± standard deviation.

permeability would allow the administered monoclonal antibodies into the extravascular space of the tumor for antibody-antigen interactions. It is also possible that such increased permeability may be contributing to the increased levels of radioactivity accumulation at the tumor site as both the anti-epiglycanin and the non-specific 49H.24 had increased tumor accumulation of radioactivity which was higher than most of the other tissues. In order to determine the retention characteristic of the administered monoclonal antibodies in the various tissues studied (table 20) the percent uptake of the injected dose remaining in the whole body per gram of tissue was calculated at the various time intervals. Most of the tissues including the blood demonstrated a clearance of both the radiotracers with time. A slight increase in kidney retention of both radiotracers could be due to the renal clearance of any free radioiodide which became available as the protein was metabolized. The tumor (figure 24) was the only tissue that showed relatively marked retention characteristics in comparison to the rest of the tissues for both the administered monoclonal antibodies. To determine the specificty of the anti-epiglycanin binding to the various tissues and the tumor, the specificity index was calculated and the results are presented in table 21. The specificity index calculated for anti-epiglycanin was found to be the highest for the tumor only at 48 hours post-injection. At the earlier time periods, other organs were found to have a specificity index that were slightly higher than the tumor. Even at 48 hours, the specificity index calculated for the tumor was not significantly higher than some of the other tissues. It could be possible that the non-specific 49H.24 control might be in fact binding the TA3/Ha cells *in vivo* although it has been reported that this non-specific monoclonal antibody used has no affinity for the TA3/Ha cells *in vitro*[274], thus resulting in the low specificity index calculated (table 21). To further check on the specificity of anti-epiglycanin for the TA3/Ha tumor *in vivo*, a different non-specific IgM antibody was used in a 24 hour biodistribution study.

Table 20: Percent of the remaining whole body radioactivity per gram of tissue[1].

	I-125 labeled anti-epiglycanin				I-131 labeled 4911.24			
Tissues	6 hours	24 hours	48 hours	72 hours	6 hours	24 hours	48 hours	72 hours
Blood	13.77	11.46	8.29	4.88	17.27	14.53	10.74	7.77
Salivary glands	27.02	21.38	8.09	7.32	23.04	16.65	7.64	6.25
Spleen	8.76	8.90	5.33	4.95	4.69	5.14	4.85	4.25
Stomach	25.36	21.24	7.60	7.72	23.64	16.28	6.21	5.95
GIT	3.70	3.41	1.90	1.64	4.25	3.10	2.10	2.14
Kidneys	7.30	7.67	8.51	7.86	9.72	11.42	16.47	15.29
Muscle	1.27	1.33	0.82	1.16	1.31	1.34	1.46	1.51
Lungs	2.80	6.53	4.60	3.24	9.03	7.28	5.78	4.35
Liver	8.27	6.25	4.51	3.97	8.25	7.86	10.85	10.54
TA3/Ha tumor	9.70	22.33	34.16	29.30	8.69	15.55	19.04	15.46

[1] n = 8

Table 21: Specificity index calculated for anti-epiglycanin[1,2].

Tissues	6 hours	24 hours	48 hours	72 hours
Salivary glands	1.39	1.62	1.42	1.81
Spleen	2.39	2.23	1.58	1.80
Stomach	1.37	1.68	1.68	2.06
GIT	1.10	1.40	1.22	1.19
Kidneys	0.91	0.85	0.70	0.81
Muscle	1.15	1.29	0.74	1.23
Lungs	1.10	1.15	1.09	1.18
Liver	1.22	0.99	0.58	0.59
TA3/Ha Tumor	1.38	1.78	2.29	3.12

[1] $n = 8$

[2] The values obtained were calculated using 49H.24 antibodies as the non-specific localization control antibody.

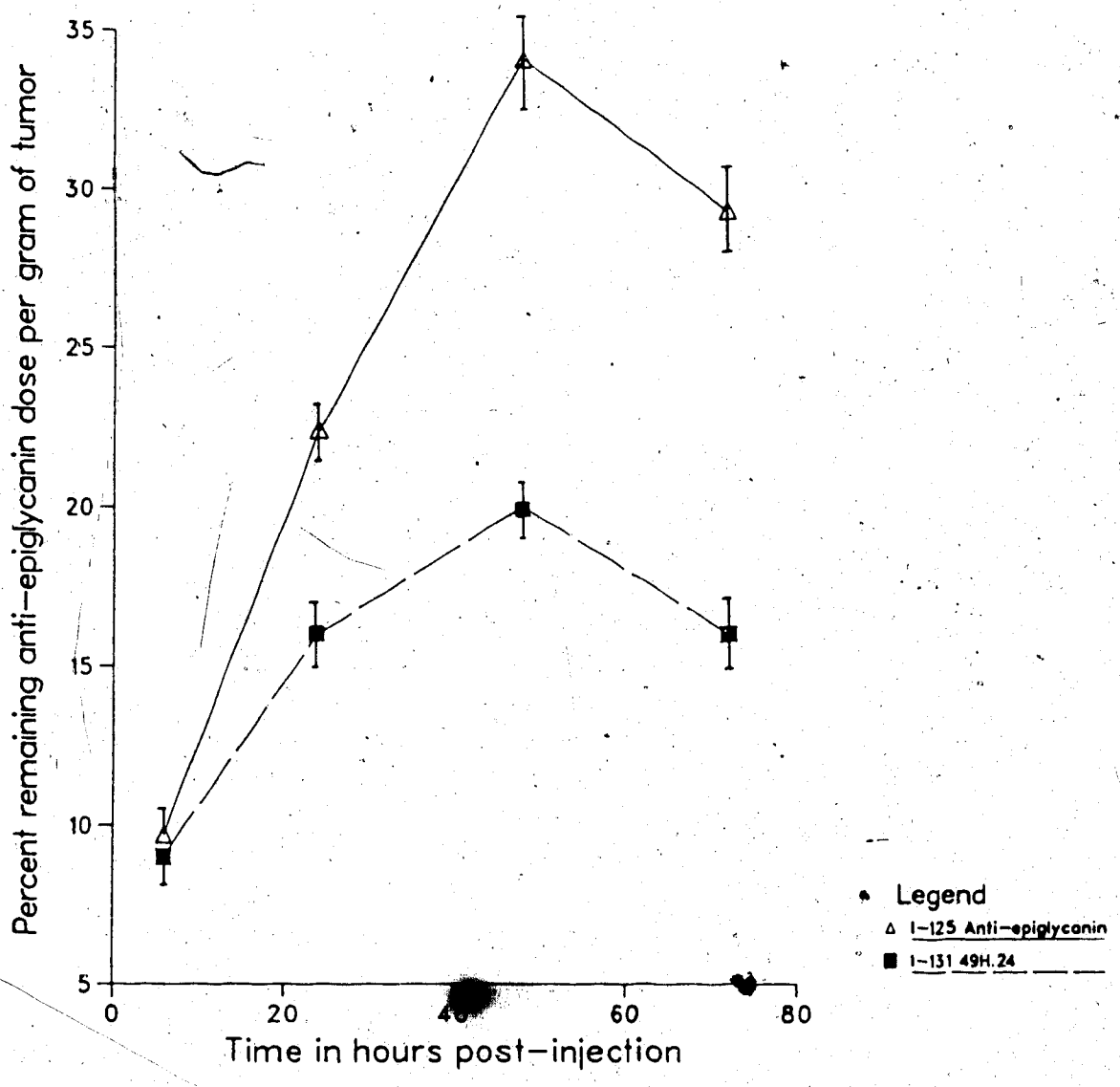


Figure 24 Percent of the remaining whole body radioactivity per gram of tumor tissue.

Biodistribution of I-125 anti-epiglycanin and I-131 nonspecific murine myeloma IgM antibodies

A chromatographically purified mouse myeloma IgM (Miles Scientific, IL.) that was a combination of equal amounts of myeloma IgM isolated from ascites fluid generated by the tumor line TEPC 183 and MOPC 104E was used as another nonspecific IgM antibody control to compare the localization of anti-epiglycanin monoclonal antibody in the TA3/Ha murine animal tumor model. The results of this 24 hour biodistribution study are presented in table 22 where the uptake of anti-epiglycanin monoclonal antibody is compared with the non-specific IgM antibody at the per gram tissue and at the whole organ basis. The results indicate a low overall uptake of anti-epiglycanin by the tissues except for the blood and tumor. Whole body retention of the radiolabeled anti-epiglycanin at 24 hours post-injection was calculated to be about 8.0%. Tissue to blood ratios calculated for both the proteins administered are given in table 23. Due to the high blood retention of ^{125}I-anti-epiglycanin, the tissue to blood ratios calculated were found to be low for most organs. Tumor to blood ratio for the ^{125}I anti-epiglycanin was calculated to be 1.58 $\pm$ 0.58 at time of sacrifice. The non-specific IgM was found to have most of its remaining radioactivity in the blood, liver and tumor. There was about 16.0% of the injected non-specific IgM dose remaining in the animal at the time of sacrifice, 24 hours post-injection. In comparison with the tissue uptake of anti-epiglycanin, the tissue levels of the nonspecific control IgM antibody were observed to be higher, probably as a result of the prolonged retention of the control IgM in the blood. Tissue to blood ratios calculated for the non-specific IgM (table 23) was found to give almost similar figures as that of the anti-epiglycanin in most of the tissues although the tumor to blood ratio was somewhat higher for anti-epiglycanin than for the IgM control. The specificity index was then calculated to determine the specific localization of the anti-epiglycanin in the various tissues especially the tumor. Results of this calculation are also given in table 23

Table 22: Biodistribution of ^{123}I anti-epiglycanin in strain A mice[1] subcutaneous TA3/Ha solid tumors[2] at 24 hours post-injection.

	Anti-epiglycanin		IgM control	
Tissues	%dose/gram	%dose/organ	%dose/gram	%dose/organ
Blood	0.90±0.11	0.96±0.12	2.52±0.45	2.80±0.60
Salivary glands	0.28±0.07	0.04±0.01	0.82±0.05	0.13±0.01
Spleen	0.66±0.30	0.17±0.07	0.56±0.19	0.15±0.06
Stomach*	0.53±0.15	0.22±0.05	1.33±0.51	0.54±0.17
GIT*	0.27±0.08	0.69±0.19	0.46±0.20	1.15±0.49
Kidneys	0.46±0.06	0.15±0.02	2.40±0.37	0.75±0.05
Muscle	0.15±0.09	...	0.79±0.36	...
Lungs	0.44±0.06	0.07±0.01	1.12±0.25	0.18±0.04
Liver	0.47±0.07	0.62±0.09	0.93±0.09	1.22±0.12
TA3/Ha tumor	1.42±0.50	1.02±1.55	2.20±0.57	1.45±1.83

[1] $n = 6$.

[2] Values are expressed as the mean percent uptake ± standard deviation.

* including contents

Table 23: Tissue to blood ratios calculated at 24 hours post-injection[1]

Tissues	Anti-epiglycanin	IgM control	Specificity index[2]
Salivary glands	0.31±0.06	0.33±0.07	0.92
Spleen	0.71±0.26	0.23±0.07	3.12
Stomach•	0.60±0.20	0.52±0.14	1.14
GIT•	0.30±0.08	0.18±0.06	1.66
Kidneys	0.52±0.09	0.97±0.16	0.53
Muscle	0.16±0.07	0.32±0.16	0.49
Lungs	0.49±0.08	0.45±0.08	1.10
Liver	0.53±0.09	0.37±0.05	1.41
TA3/Ha tumor	1.58±0.78	0.88±0.19	1.91

[1]Values are expressed as the mean ratio (n = 6) ± standard deviation for the tissue:blood ratios.

[2]The specificity index was calculated using the IgM control as the non-specific localization agent.

• including contents

showing that anti-epiglycanin was not highly specific in its tumor localization. The value obtained for the tumor was found not to be significantly higher than for most of the other tissues.

In comparison to the previous biodistribution study, the two non-specific IgM proteins used were found to behave similarly in most of the tissue uptake at 24 hours post-injection. Both proteins were found to exhibit prolonged retention times that were about twice that of anti-epiglycanin, and both were taken up more by the liver, blood and tumor in the TA3/Ha animal tumor model. From both the biodistribution studies done, it was seen that both of the non-specific IgM antibodies used gave almost similar results and both of these IgM antibodies are nonspecific for the TA3/Ha tumor. Hence, both the localization indices calculated using 49H.24 and the non-specific IgM antibodies as the non-specific localization controls should be valid and not artifacts due to possible selective uptake of either of the control IgM antibodies used. Both the values of the specificity indices calculated for anti-epiglycanin using the two different IgM controls were found to be somewhat similar for most of the tissues including the tumor. As seen from both the biodistribution studies, the non-specific controls behaved almost similarly to the anti-epiglycanin monoclonal antibody in terms of tissue uptake. Both of the non-specific IgM antibodies had high blood concentrations and higher tumor uptake than the other tissues which was similar to the behaviour of anti-epiglycanin. Although there was a higher specificity index calculated for the tumor than the rest of the tissues, it could not be fully ascertained that the accumulation of the anti-epiglycanin at the tumor site was totally due to the specific anti-epiglycanin-TA3/Ha tumor binding. The percent of the remaining injected dose contained per gram of tissue calculated for anti-epiglycanin and 49H.24 showed that both the proteins administered were retained to an extent by the tumor (figure 24) while the rest of the organs were found not to retain much of either proteins except for the kidneys (table 20). Tumor to blood ratios and tumor to liver ratios for

anti-epiglycanin at 24 hours post-injection was calculated to be 1.58 and 2.37 respectively, while the same ratios calculated for the non-specific IgM antibody was 1.91 and 2.37. Reports have indicated that with the present conventional imaging techniques, a minimum tumor to non-tumor concentration ratio of 8:1 is required for satisfactory definition of a malignant lesion[190]. With lower tumor to non-tumor ratios, the technique of background subtraction may be necessary and reports of false positive results that have been generated from background subtracted imaging procedures have been cited in the literature[190].

The exact mechanism of localization of anti-epiglycanin at the tumor site has yet to be determined although *in vitro* studies showed that the anti-epiglycanin bound to the TA3/Ha tumor cells. Unfortunately, the biodistribution results obtained indicated that the localization of anti-epiglycanin at the tumor site could not be totally due to specific antibody-antigen interaction. There is some indication, by virtue of the retention of the non-specific 49H.24 at the tumor site and the higher tumor uptake of both the non-specific IgM antibodies in comparison to the other tissues, that accumulation of anti-epiglycanin at the tumor site could partially be due to entrapment of the high molecular weight antibody in the extravascular space of the tumor, "sticky cells" and non-specific binding of the antibody with its Fc portion to Fc receptors that could be present at the tumor site.

Whole body gamma scintigraphy

Whole body gamma scintigraphic imaging was performed on A/J mice bearing the TA3/Ha solid tumor in the right flank at 6, 24 and 48 hours post-injection of ^{125}I-anti-epiglycanin. Figures 25 to 27 illustrate the scintigraphic images acquired on a floppy disk with the aid of the ADAC CAM II clinical acquisition processing unit. The scans show the uptake of radioiodinated anti-epiglycanin at the different periods post-injection of the radiotracer. At 6 hours post-injection (figure 25), high levels of radioactivity were seen throughout the whole body with higher accumulation of activity

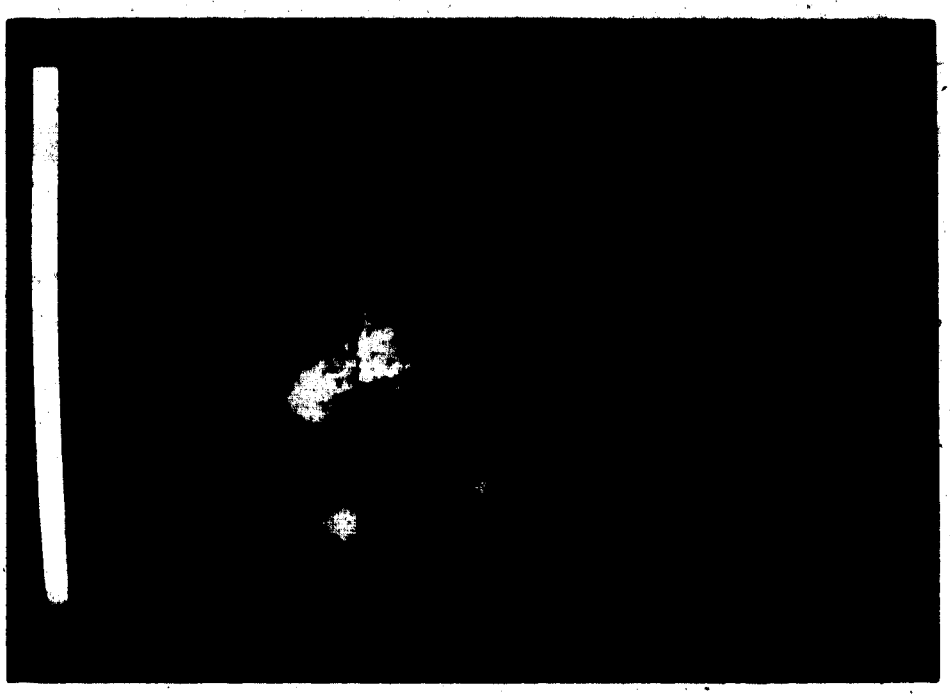


Figure 25 Whole body gamma scintigraphic image of an A/J mouse bearing the TA3/Ha solid tumor 6 hours post-injection of 2 MBq ^{125}I-anti-epiglycanin.

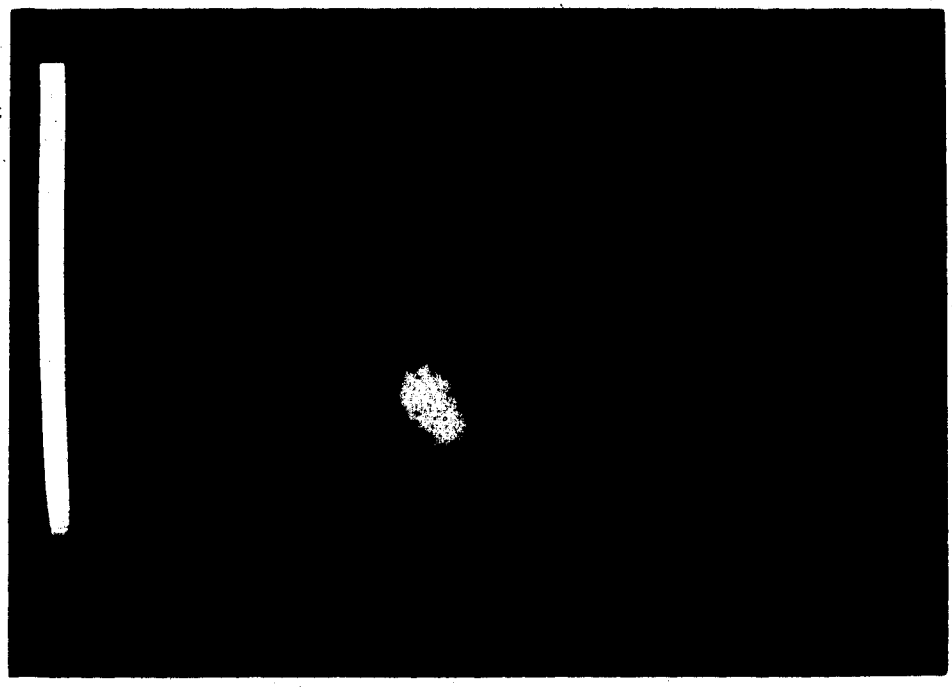


Figure 26 Whole body gamma scintigraphic image of an A/J mouse bearing the TA3/Ha solid tumor 24 hours post-injection of 2 MBq ^{125}I-anti-epiglycanin.

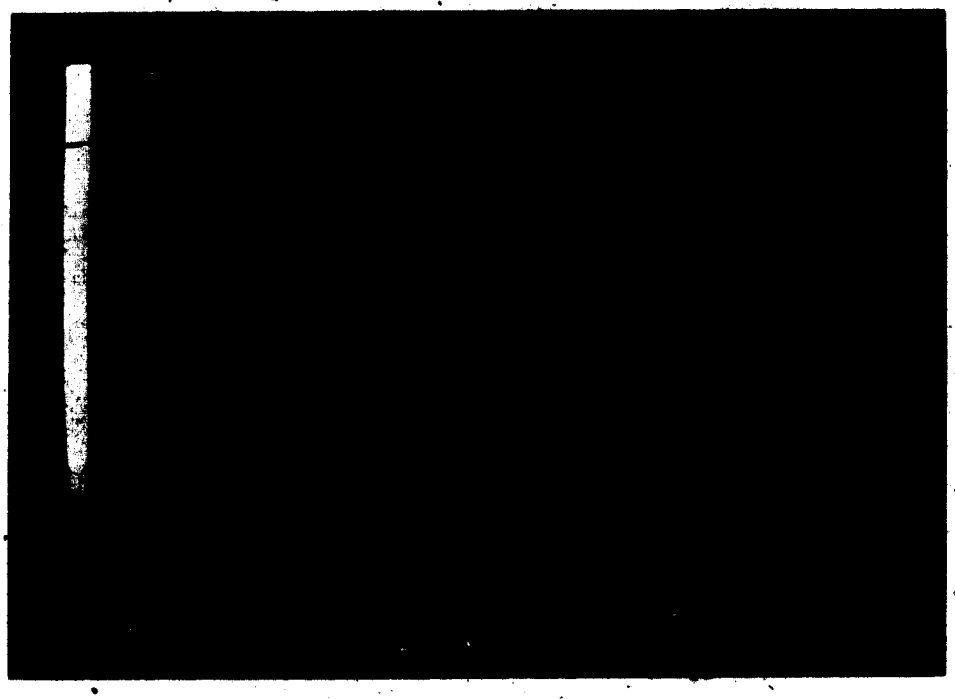


Figure 27 Whole body gamma scintigraphic image of an A/J mouse bearing the TA3/Ha solid tumor 48 hours post-injection of 2 MBq ^{125}I-anti-epiglycanin.

noted in the salivary glands, kidneys, bladder, liver and tumor. At 24 hours post-injection, there was still some background activity seen in the whole body which was probably due to the ^{125}I-anti-epiglycanin circulating in the blood. Radioactivity accumulation in the kidneys, bladder and salivary glands was not seen at this time period (figure 26). It would seem that the areas of increased radioactivity seen in the kidneys, bladder and salivary glands seen at 6 hours post-injection was most probably due to free radioiodide from the *in vivo* breakdown of the label with rapid excretion. The 24 hour scan showed intense localization of radioactivity at the tumor site with radioactivity in the liver still apparent at 24 hours post-injection. The scintigraphic image obtained at 48 hours post-injection (figure 27) showed intense localization of the radioiodinated anti-epiglycanin at the tumor site. The liver showed less accumulation of the radiotracer at this time period in comparison to the scan obtained at 24 hours post-injection. Uptake of the radioiodinated anti-epiglycanin seen in the liver at the various time periods post-injection could possibly be due to the immune complexes formed by the interaction of shed epiglycanin from the tumor with the administered anti-epiglycanin monoclonal antibody with the resultant uptake of the complexes by the reticuloendothelial system. There could also be some non-specific binding of the Fc portion of the IgM antibody to the hepatocytes and monocytes resulting in increased liver uptake. The presence of circulating epiglycanin molecules shed from the tumor did not appear to interfere with the localization of the anti-epiglycanin antibody at the tumor site. There was intense localization of radioactivity at the tumor site at all the time periods studied (figures 25 to 27) while the background radioactivity in the circulation and the other organs including the liver was seen to clear with time. Scintigraphic imaging, however, of the animals at 24 hours and 48 hours post-injection took a very long time due to the low levels of radioactivity remaining in the mouse. Accumulation of a total of 25 000 counts at 24 hours post-injection took more than 30 minutes while accumulation of 10 000 counts at 48 hours post-injection took close to an

hour even though more than 2 MBq of radioactivity was administered. As seen in the biodistribution studies, there was a rapid body clearance of ^{125}I-anti-epiglycanin with about 8-12% of the injected dose remaining at 24 hours post-injection. Most of the radioactivity seen in the scans was localized at the tumor site at 24 and 48 hours post-injection. The apparently short biological half-life of anti-epiglycanin combined with the intense localization at the tumor site and low uptake of radioactivity by the other organs except the liver, indicates that anti-epiglycanin may be a useful radioimmunoimaging agent for this animal tumor model. The whole body radiation dose would also be low due to the low retention of anti-epiglycanin by non-tumor organs and the whole body.

In spite of the positive scans obtained with the radioiodinated anti-epiglycanin monoclonal IgM antibody, much more work has to be done to determine the potential of this agent as a radioimmunoimaging agent. Biodistribution studies done with the anti-epiglycanin and the two different non-specific controls indicate that there was some degree of non-specific localization of the anti-epiglycanin at the tumor site. The extent of the non-specific localization has yet to be determined for the anti-epiglycanin as data indicate that there was rather low absolute uptake of this agent by the tumor and also the tumor to non-tumor organ ratios were low. Thus, if the degree of non-specific localization is high, then further studies should be attempted to increase the specificity such as the use of smaller antibody fragments without the presence of the Fc portions. Retention of radioactivity at the TA3/Ha tumor site was not unique to anti-epiglycanin as the control 49H.24 antibody was also seen to be retained by the tumor. It appears that the biodistribution data obtained for the anti-epiglycanin does not look very favourable even though the *in vitro* cell binding studies and the gamma scintigraphic images appear promising. Furthermore, radioiodination of the anti-epiglycanin monoclonal IgM antibody was found to cause the antibody to form microaggregates with more than 80% of the total radioactivity settling out under

prolonged ultracentrifugation. The IgG class and its F(ab'), fragments of the anti-epiglycanin monoclonal antibodies may give a more promising view on the potential of anti-epiglycanin as a tumor localizing agent. Tissue permeability of IgG antibodies is greater than that of IgM antibodies[197]. Furthermore, F(ab'), fragments of the IgG antibodies, devoid of the Fc portion, have been found to be more efficient in penetrating tumor tissues than the intact IgG antibody, and in combination with the fragments' accelerated clearance from the blood may make the F(ab'), fragments more preferable over the intact antibody for tumor localization[197].

Anti-epiglycanin being a murine antibody may also suffer some of the clinical limitations of murine antibodies that have been used[193,194]. Murine antibodies will be foreign to a human body if administered and this could result in the stimulation of an immune response from the host which may complex and deactivate the antibody. Another more severe possibility is that the murine antibody may cause serum sickness and/or an anaphylactic shock upon the administration to a human body[193,194]. A major problem that is faced with most monoclonal antibodies is the phenomena of antigenic modulation[196,198-200]. This poses a problem in that using an antibody for the detection of a tumor which may induce antigenic modulation could result in the tumor becoming resistant should the same antibody be later used for treatment and may also impair any further monitoring of the tumor state[190].

Biodistribution of a mixture of anti-epiglycanin and peanut lectin

Results of individual studies performed on each of the proteins, anti-epiglycanin and the peanut lectin, showed that they each exhibited some binding to the TA3/Ha tumor both *in vivo* and *in vitro*. *In vitro* studies showed that each of the proteins bound to the TA3/Ha cells and the presence of both proteins with the TA3/Ha tumor cells did not affect the binding of each of the proteins to the tumor cells. Hence, it was postulated that the peanut lectin and anti-epiglycanin may possess different binding sites on the TA3/Ha cells and neither protein

bore any hinderance to the binding activity of the other. This phenomena was further investigated in the TA3/Ha animal tumor model to determine whether the simultaneous administration of both proteins would have any effect on the tumor uptake of these agents. Table 24 gives the results of the study on the peanut lectin and table 25 gives the results obtained for anti-epiglycanin. Both tables 24 and 25 give the results comparing the uptake of the agent administered alone to that administered in the mixture. The table 26 gives the comparative tissue to blood ratios obtained from the study for the peanut lectin and the anti-epiglycanin. The results obtained for the peanut lectin showed no apparent difference in PNA tissue uptake between PNA administered alone or in the PNA-anti-epiglycanin mixture. No significant changes in uptake of the peanut lectin was seen. Similarly, the simultaneous injection of the peanut lectin and anti-epiglycanin did not appear to affect the localization of the peanut lectin in the tumor at all nor did it affect the localization of PNA at all the various tissues studied. Tissue to blood ratios were calculated not to be significant between the uptake of PNA administered alone or in the mixture. The same observation was made for the anti-epiglycanin as seen from the results obtained. Thus, it appears that the peanut lectin and anti-epiglycanin possess different binding sites on the same tumor as evidenced by the *in vitro* cell binding studies and from this biodistribution study. Anti-epiglycanin may not be specific for the immunodominant structure of the T-antigen as neither the the presence of the peanut lectin nor galactose seem to affect the binding activity of anti-epiglycanin to the TA3/Ha tumor cells. The presence of galactose was also found not to inhibit the binding of anti-epiglycanin to neuraminidase treated red blood cells. From the results of this study, the concept of using a "cocktail" or mixture of tumor localizing agents that have binding affinities for the same tumor but have different binding sites to possibly further potentiate the defination and delineation of a tumor is potentially another approach to tumor detection especially if the tumor exhibits low expression of single antigens. The use of radioiodinated peanut lectin alone in this animal tumor model has been found to give high enough tumor resolution without the need of any data manipulation or background subtraction. The use of this PNA and anti-epiglycanin mixture

Table 24: Tissue uptake of ^{125}I-PNA administered alone and in the PNA-anti-epiglycanin mixture[1,2].

	PNA alone[3]		PNA in mixture with anti-epiglycanin[4]	
Tissues	%dose/gram	%dose/organ	%dose/gram	%dose/organ
Blood	0.75±0.41	0.98±0.46	1.08±0.78	1.50±1.05
Salivary glands	9.94±4.93	1.61±0.80	9.71±2.96	1.86±0.64
Spleen	1.68±0.23	0.54±0.04	2.71±0.34	0.67±0.14
Stomach*	3.65±2.18	1.10±0.42	3.21±2.28	0.99±0.59
GIT*	0.82±0.43	2.04±0.82	0.92±0.60	2.56±1.49
Kidneys	15.77±4.52	5.71±1.39	19.25±5.84	7.18±1.96
Muscle	0.27±0.06	...	0.33±0.15	...
Lungs	3.29±1.46	0.61±0.23	2.81±0.72	0.47±0.10
Liver	2.39±0.43	3.15±0.69	3.17±0.74	4.19±0.59
TA3/Ha tumor	3.80±0.63	1.75±0.86	5.21±2.04	1.51±1.11

[1] $n = 6$.

[2] Values are expressed as the mean percent uptake ± standard deviation.

[3] 1.0 μg PNA (150 kBq ^{125}I PNA)

[4] 1.0 μg PNA with 1.0 μg (150 kBq ^{131}I) anti-epiglycanin.

* including contents

Table 25: Tissue uptake of ^{125}I-anti-epiglycanin administered alone and in the PNA-anti-epiglycanin mixture[1,2].

Tissues	Anti-epiglycanin alone[3]		Anti-epiglycanin in mixture with PNA[4]	
	%dose/gram	%dose/organ	%dose/gram	%dose/organ
Blood	2.21±0.85	2.78±1.12	2.92±0.53	4.11±0.83
Salivary glands	3.87±1.24	0.55±0.15	2.52±1.46	0.47±0.28
Spleen	0.86±0.35	0.24±0.12	1.01±0.14	0.27±0.07
Stomach*	2.15±0.88	0.67±0.34	1.96±1.33	0.63±0.42
GIT*	0.64±0.41	1.53±0.29	0.68±0.32	1.93±0.85
Kidneys	1.23±0.45	0.44±0.16	1.14±0.30	0.43±0.11
Muscle	0.31±0.17	...	0.31±0.10	...
Lungs	1.99±0.53	0.20±0.09	1.28±0.44	0.23±0.10
Liver	1.04±0.39	1.26±0.40	1.10±0.19	1.45±0.15
TA3/Ha tumor	2.76±0.84	1.77±1.10	2.53±0.83	0.90±0.27

[1] $n = 6$.

[2] Values are expressed as the mean percent uptake ± standard deviation.

[3] 1.0 μg (150 kBq ^{131}I) anti-epiglycanin

[4] 1.0 μg ^{131}I anti-epiglycanin with 1.0 μg ^{125}I PNA (150 kBq)

* including contents

Table 26: Comparative tissue to blood ratios for ^{125}I-PNA and ^{131}I-anti-epiglycanin mixture[1].

Tissues	PNA	PNA in mixture	Anti-epiglycanin	Anti-epiglycanin in mixture
Salivary glands	14.78±4.92	11.91±5.09	1.49±0.23	0.83±0.34
Spleen	2.91±1.45	4.05±2.81	0.42±0.26	0.36±0.07
Stomach	4.71±0.99	2.90±0.83	0.91±0.52	0.63±0.35
GIT	1.09±0.06	0.89±0.04	0.27±0.12	0.23±0.07
Kidneys	28.83±9.04	24.40±8.09	0.56±0.15	0.39±0.04
Muscle	0.45±0.20	0.38±0.12	0.14±0.07	0.10±0.02
Lungs	5.71±2.08	4.24±1.23	0.55±0.16	0.45±0.16
Liver	4.21±2.26	4.32±2.74	0.48±0.10	0.38±0.04
TA3/Ha tumor	6.49±3.20	7.04±2.83	1.34±0.52	0.87±0.25

[1]Values are expressed as the mean tissue:blood ratio ± standard deviation.

would be of potential use in the detection of tumors that have low expressions of the T-antigen to possibly enhance the tumor image without the need of data manipulation such as background subtraction as both the peanut lectin and anti-epiglycanin have short biological half-lives resulting in a fast clearance of background activity.

Summary and conclusions

1. Affinity purified peanut lectin was routinely labeled with efficiencies of about 50% using the iodogen method and the radiochemical stability of the final product was greater than 95% for at least a week post-iodination when stored at 4° C.
2. *In vitro* protein binding studies resulted in the radioiodinated peanut lectin exhibiting specific binding affinity for the immunodominant disaccharide, β-D-Gal (1→3)GalNAc, of the asialo GM-1 synsorb, neuraminidase treated red blood cells and the TA3/Ha mammary adenocarcinoma cells as well as their selected organ specific metastatic variants, but not normal red blood cells or the non-T antigen expressing EL4 tumor cells. The binding activity of I-125 labeled PNA could be readily inhibited by the presence of galactose.
3. Biodistribution studies found that ^{125}I-PNA administered in the range of 0.1 μg to 1.0 μg per 20 g mouse gave the highest localization index for the tumor with lower uptake of the radiotracer by the other organs.
4. Whole body gamma scintigraphic imaging with ^{125}I-PNA in strain A mice bearing a solid TA3/Ha tumor revealed good tumor delineation by 24 hours post-injection without the need for blood background subtraction.
5. Biodistribution studies with ^{125}I-PNA in mice bearing the TA3/Ha tumor at various time periods revealed high tumor uptake of the radiotracer and tumor to non-tumor tissue ratios were high. Specific tumor localization indices of ^{125}I-PNA were 8.6 at 24 hours and 49.5 at 48 hours post-injection using an F(ab')$_2$ protein IgG fragment as a non-specific control. The kidneys were the only other organs to demonstrate preferential uptake of ^{125}I-PNA and it is suspected that the tubules of the kidneys may be involved in a unique mechanism of binding and excreting the peanut lectin when urine analysis found that PNA was excreted in an active form.
6. Organ specific metastatic variants of the TA3/Ha mammary adenocarcinoma have been selected and made available by Dr. B.M. Longenecker, Department of Immunology,

University of Alberta. High preferential uptake of ^{125}I-PNA by the tumor infiltrated organs was seen *in vivo*. There was about 7 to 8 times as much uptake of ^{125}I-PNA in these organs compared to those of the controls and the difference in uptake was calculated to be statistically significant.

7. I-125 PNA was able to penetrate and distribute throughout the tumor mass of the TA3/Ha metastatic liver variant *in vivo* and is retained by the tumor mass as evidenced by the autoradiograms taken of a liver tissue section of a mouse bearing the TA3/Ha metastatic liver variant . PNA receptors were evident throughout the TA3/Ha liver metastatic tumor mass as seen in the tumor infiltrated liver section stained by FITC-PNA.

8. Based on all the *in vitro* and *in vivo* studies done, radioiodinated PNA has been established as a potential radioimaging agent of T antigen expressing solid subcutaneous tumors as well as metastatic lesions.

9. Anti-epiglycanin IgM monoclonal antibody demonstrated high binding affinity for the TA3/Ha mammary adenocarcinoma tumor cells and neuraminidase treated red blood cells but not normal red blood cells *in vitro*. The binding activity of anti-epiglycanin was not affected by the presence of galactose or PNA. Ultracentrifugation of the anti-epiglycanin IgM antibody for prolonged periods after radioiodination resulted in about 80% of the total radioactivity settling out. Preliminary biodistribution studies found the anti-epiglycanin monoclonal antibody not to accumulate significantly more than the nonspecific IgM protein controls at the tumor site although gamma scintigraphic imaging demonstrated intense localization of radioactivity at the tumor site. Further studies with the IgG class or its $F(ab')_2$ fragments of the anti-epiglycanin monoclonal antibody line may be more promising.

10. *In vitro* and *in vivo* studies indicated that PNA and anti-epiglycanin do not share the same binding sites on neuraminidase treated red blood cells and the TA3/Ha tumor cells. Biodistribution studies demonstrated that the presence of either anti-epiglycanin

or PNA did not affect the binding affinity each had for the TA3/Ha tumor cells.

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