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RADIOIODINATED ANTI-T ANTIBODIES AND PEANUT LECTIN
FOR DETECTION OF T ANTIGEN EXPRESSING TUMOURS

by

 PAMELA L. ZABEL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
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RADIOIODINATED ANTI-T ANTIBODIES AND PEANUT LECTIN FOR
DETECTION OF T₁ ANTIGEN EXPRESSING TUMOURS

submitted by PAMELA L. ZABEL in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in PHARMACEUTICAL SCIENCES (Radiopharmacy).

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DEDICATION

To My Parents

ABSTRACT

The Thomsen-Friedenreich (T) antigen, containing the β -D-Gal-(1 \rightarrow 3)- α -D-GalNAc moiety, is exposed in reactive form on many human adenocarcinomata, but not on the corresponding benign tissues. Anti-T antibodies and peanut lectin have strong affinities for the T antigen and bind preferentially to certain malignant tissues. The potential utility of radiolabelled anti-T antibodies and peanut lectin (PNA) as tumour localizing agents was investigated in an animal-tumour model system using a mouse lymphoma (RI) shown to bind fluorescein-labelled PNA and anti-T antibodies in vitro.

Preliminary investigations involved a rabbit IgG preparation of anti-T antibodies in which a protected radioiodination of the protein was gradually developed. The resulting ^{125}I -anti-T was utilized to study the in vitro binding and in vivo tumour localization of the radiolabelled protein. Although excellent binding to a synthetic T immunoabsorbent was evident, in vitro binding investigations did not indicate a particularly strong binding affinity for T antigen glycoproteins expressed on the cell surfaces of RI tumour cells and neuraminidase-treated red blood cells. This was supported by in vivo localization studies, in which the radiolabelled anti-T antibody preparation showed some tumour localization but to an extent that was not sufficiently greater than a nonspecific control IgG antibody. Therefore the emphasis of the investigations

were placed on another T-antigen avid protein, the peanut lectin.

The peanut lectin protein was radiolabelled with ^{125}I to provide a radiopharmaceutical which maintained its biological activity following iodination and remained relatively radiochemically stable, with less than 5% deiodination occurring after 2 weeks at 4°C . Analysis of the in vitro binding affinity of radiolabelled peanut lectin revealed an avid, and specific, in vitro binding of the peanut lectin for cells and immunoadsorbents expressing the T antigen. The carbohydrate binding specificity was confirmed by investigating the binding of radiolabelled peanut lectin to immunoadsorbents of various carbohydrate specificities. Tissue biodistribution studies in RI lymphoma-bearing mice revealed that the radioiodinated lectin localized well in the tumours and was eliminated rapidly from the blood. Clear images of tumours were obtained, in serial scintigraphic imaging, by 24 and 48 hours post-injection. No blood subtraction was necessary. Biodistribution studies revealed tumour: blood ratios of radioactivity in the mice were 6:1 (24 hr.) and 17:1 (48 hr.), and tumour:muscle ratios were 34:1 (24 hr.) and 40:1 (48 hr.). Rapid in vivo breakdown of ^{125}I -PNA led to some localization of free iodide in the kidneys, stomach, thyroid and salivary glands.

The in vivo specificity of tumour localization of peanut lectin was investigated by comparing its biodistribution to a non-specific protein, $\text{F}(\text{ab}')_2$ fragment

of normal gamma globulin in mice bearing tumours which express the T antigen. Less than ten per cent of the tumour localization, based on tumour: blood ratios, of peanut lectin could be attributed to non-specific macromolecular accumulation in the tumours and this preferential PNA tumour localization was not paralleled in a mouse tumour model in which the cells did not express the T antigen. The investigations indicate a potential usefulness of radiolabelled peanut lectin for the detection and delineation of tumours and their metastases which express the T antigen on their cell surfaces.

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INTRODUCTION

Many cellular activities are conveyed at the cell surface and often a great deal of information about the state of a cell can be discerned from its cell surface glycoproteins. Neoplastic transformations have been shown to modify some cell surface properties such as the expression of various carbohydrate chains, thereby allowing differentiation between normal and malignant cells. Two classes of proteins, lectins and carbohydrate-specific antibodies, are able to distinguish the numerous carbohydrates and then bind specifically and reversibly with their corresponding sugar residues. Both antibodies and lectins have therefore been invaluable in investigations of the carbohydrate nature of cell surfaces.

The Thomsen-Friedenreich (T) antigen has recently stimulated interest as a tumour-associated marker since structures with this specificity have been found on certain human carcinomata but not on other diseased or on healthy tissues. The T antigen immunodeterminant structure is β -D-Gal(1+3)-GalNAc and two proteins which bind very avidly and specifically to this disaccharide are the peanut (Arachis hypogoea) lectin and anti-T antibodies.

These T antigen-avid proteins could be radiolabelled to provide biologically active proteins with a very specific affinity for cells expressing the T antigen. It was therefore the aim of this project to develop an animal tumour model and to test the in vivo tumour localizing capability of radiolabelled anti-T antibodies and peanut lectin for

potential utility in diagnostic radioimmuno-detection of
selected cancers.

SURVEY OF THE LITERATURE

A. TUMOUR LOCALIZING RADIOPHARMACEUTICALS

1. "Nonspecific" Tumour Imaging Agents

Nuclear medicine plays an important role for the clinical oncologist, providing a non-invasive diagnostic procedure capable of rapidly screening the entire body for cancer, if appropriate radiopharmaceuticals are available¹.

Tumour scanning has become an established method for (1) assessing the initial extent of spread (stage) of primary and metastatic lesions and (2) providing a means of evaluating the efficacy of a therapeutic procedure².

It is desirable for the radiopharmaceutical to exhibit a selective uptake in the tumour in order to achieve a tumour:background ratio, which permits detection of the neoplastic lesions. Typically, when the tumour:background ratio is 2:1, tumour lesions will only be detected if the diameter is larger than about 2 centimeters³. With improved concentration ratios, smaller lesions may be detected successfully and thus allow for earlier treatment of the cancer.

The growth of tumour masses may alter tissue physiology or displace normal tissue, leading to abnormal scans with commonly used radiopharmaceuticals⁴. These "nonspecific limited-use" agents⁴ are able to detect physiological changes, such as: (1) space occupying lesions in Tc-sulfur colloid liver images, (2) increased permeability of the blood brain barrier in ^{99m}Tc

(pertechnetate and its chelates) brain scans and (3) increased bone mineral metabolism in ^{99m}Tc -phosphate bone scans. All of these methods are unfortunately nonspecific and the scan abnormalities may also be due to nonmalignant processes⁴. In addition, only one organ or tissue system is imaged, so that different radiopharmaceuticals are required for the evaluation of various organs².

Some tumour imaging agents attempt to exploit a qualitative or quantitative difference of tumour tissue which distinguishes it from normal tissue⁴. Labelled metabolites can be tailored to a particular tumour. Thus, iodocholesterol labelled with ^{131}I is known to accumulate in cortisol-secreting adrenal tumours⁵ and ^{75}Se - selenomethionine localizes in protein synthesizing hepatomas and parathyroid adenomas⁶. Melanin-avid chloroquine analogues successfully localize in melanomas⁷ and thyroid lesions can be imaged with radioiodine⁸ or ^{99m}Tc pertechnetate. Most of these agents are not useful for routine tumour diagnosis, because they fail to interact with a broad spectrum of tumour types⁴.

Certain antibiotics have been known to affect the growth of tumours and therefore attempts have been made to label them, for use as tumour localizing agents⁹. Tetracycline, labelled with either ^{99m}Tc or ^{131}I , is concentrated somewhat in tumours but its uptake in nonmalignant tissue has precluded any extensive use⁹. Bleomycin, an anti-tumour antibiotic, has been labelled

with a wide variety of radionuclides including ^{57}Co , ^{111}In , ^{123}I , $^{99\text{m}}\text{Tc}$. Radiolabelled bleomycin localizes in a broad spectrum of neoplasms and demonstrates a rapid blood clearance (dependant upon the in vivo stability of the label)¹⁰. However, the bleomycin complexes are not tumour-specific and also localize in non-malignant lesions, such as inflammatory processes¹⁰. Some investigators have reported greater success in imaging certain tumours with radiolabelled bleomycin than with gallium-67 citrate, due to the lower abdominal uptake of the bleomycin¹⁰. In general, though, the previous interest in labelled antibiotics appears to have subsided, due to their lack of specificity¹.

Perhaps the most frequently used tumour localizing agent at the present time is ^{67}Ga , and recently its chemistry, properties and uses have been extensively reviewed¹¹. Excellent tumour visualization has been achieved with gallium for some tumour types, particularly bronchogenic carcinoma, lymphoma and Hodgkin's disease, hepatoma and melanoma. Unfortunately, other common tumours (breast, gynecological, gastrointestinal and genitourinary tract tumours) are often imaged with gallium with inconsistent success.

The biodistribution of radiogallium is relatively complex, with the liver, bone marrow, stomach, intestine, nasopharynx and salivary glands concentrating an appreciable fraction. Localization of gallium in the

gastrointestinal tract often obscures detection of neoplastic lesions in the abdominal cavity. Other disadvantages include the expense of radiogallium and the sub-optimal physical decay characteristics, which reduce the image quality. The major drawback, in the routine use of ^{67}Ga -citrate, is its lack of specificity. It accumulates in a wide variety of benign tissues and inflammatory lesions, thus complicating the diagnosis¹¹.

The major reason for the nonspecificity of the aforementioned tumour localizing agents stems largely from their mechanism of localization. The altered regional physiology of tumours, including increased vascular permeability and interstitial space and decreased lymphatic drainage, also occurs in non-malignant disease states¹². Therefore, localization in benign lesions, as well as in tumours, is to be expected for those radiopharmaceuticals in which localization is effected primarily as a result of altered microvasculature or nonspecific trapping¹².

2. Radioimmunodetection with Radiolabelled Antibodies

Neoplastic alteration of a cell is often associated with changes in the cell surface, which result in the expression of new protein or glycoprotein antigens¹³. Antibodies, which bind specifically to these neoantigens, can be radioactively labelled to provide agents for tumour imaging with the important property of specificity. Recently, a new hybrid discipline of nuclear medicine and

immunology has developed, specializing in the radio-immunodetection of cancer¹⁴.

The concept of injecting anti-tumour antibodies for diagnostic localization of neoplasms was first introduced in experimental systems by Pressman in 1957¹⁵. By virtue of the selectivity of such antibodies, they may also be potentially used as carriers of cytotoxic or chemotherapeutic drugs or therapeutic doses of radioactivity¹⁶.

Many immunopharmacological factors are involved in the distribution of a radioantibody. Each component of the system, antigen, antibody and radionuclide, has a very important contribution to the final success of tumour localization. A summary of important factors in radio-immunodetection have been discussed by various investigators¹³⁻¹⁹ and are summarized in Table I.

Initial studies into the use of antibodies as tumour-localizing agents indicated that these agents suffered from various limitations. ¹³¹I-rabbit antibody to human fibrinogen, showed promising results but lacked tumour specificity²⁰⁻²¹. Antibodies produced against single cell homogenates, such as renal cell carcinoma, requires the production of antibodies to individual tumours and tumour types. This must be followed by extensive work-up to remove cross-reacting antibodies²². The production of different antibody preparations for each individual patient is not practical, from the viewpoint of time-cost effectiveness and patient delay¹⁷.

TABLE 1

Major Considerations for Successful Radioimmunodetection of Tumours

Component	Required Characteristic
antigen and tumour characteristics	<ol style="list-style-type: none"> 1) <u>accessibility of antigenic site</u> <ul style="list-style-type: none"> - antigen must be present on cell surface, rather than intracellularly - antigenic-site not blocked by host antibodies 2) <u>tumour specificity of antigen marker</u> <ul style="list-style-type: none"> - antigen present only on primary and metastatic tumours and not on benign or normal tissues 3) <u>long retention of antigen on cell surface</u> <ul style="list-style-type: none"> - antibody retention time at tumour sufficient for imaging - antigens shed from tumour may interact with antibodies in circulation 4) <u>localization extent possible in tumour</u> <ul style="list-style-type: none"> - perfusion, permeability, extravascular space and lymphatic drainage of particular tumours sufficient to allow for antibody localization
antibody (or lectin)	<ol style="list-style-type: none"> 1) <u>consistent specificity; reactivity only with tumour antigen of interest</u> <ul style="list-style-type: none"> - pure antigen required to produce antibodies of specific reactivities - production, selection and purification methods capable of producing specific antibodies 2) <u>nonspecific localization is controlled or accounted for</u> <ul style="list-style-type: none"> - simultaneous injection of nonspecific antibody labelled with a different isotope 3) <u>high tumour/background ratio for good image contrast</u> <ul style="list-style-type: none"> - fast blood clearance - low binding to background tissue 4) <u>nontoxic and nonallergenic</u> <ul style="list-style-type: none"> - repeated injections of foreign proteins could induce anaphalaxis
labelling or imaging technique	<ol style="list-style-type: none"> 1) <u>choice of radionuclide - chemical and physical properties</u> <ul style="list-style-type: none"> - appropriate effective half-life of radiopharmaceutical - radionuclide amenable to scintigraphic techniques - radiation dose within acceptable limits 2) <u>labelling technique</u> <ul style="list-style-type: none"> - required levels of specific activity are attained - biological activity retained following radiolabelling procedure - radiochemical stability 3) <u>special imaging techniques</u> <ul style="list-style-type: none"> - system capable of subtraction methods for nonspecific localization and blood pool activity (eg. $^{99m}\text{TcO}_4^-$ and ^{99m}Tc-human serum albumin)

Footnote: Table compiled from information contained in references 13-19.

Great enthusiasm has recently been expressed in tumour-associated markers such as oncofetal antigens (carcino-embryonic antigen and alpha-fetoprotein) as well as ectopic hormone markers (human chorionic gonadotropin)²¹. These tumour-associated markers are frequently not tumour specific as they may also be associated with non-neoplastic diseases²¹.

The carcinoembryonic antigen (CEA) is a well studied oncofetal antigen, discovered in 1965 by Gold and Freedman²³. It is a component of embryonic and fetal gut and is also found in human gastrointestinal (GI) carcinomas. CEA has been subsequently found in a variety of normal as well as malignant tissues²⁴. Nevertheless, quantitatively increased plasma CEA levels have been found to be clinically significant in most GI cancers²⁴. Goldenberg et al²⁴ used ¹²⁵I- and ¹³¹I- labelled goat anti-CEA successfully to localize xenografts of human colonic carcinoma in hamsters. A delay of 4 to 10 days post-injection was required for optimum tumour localization, due to a slow clearance of background radioactivity by the blood and non-tumour tissues²⁴.

Several investigators have already utilized an affinity purified anti-CEA clinically for the radioimmuno-detection of cancer²⁵⁻²⁸. When anti-CEA was used for radioimmunodetection in 142 patients with a proven history of epithelial cancer (colorectal, ovarian, cervical and lung) the overall sensitivity (true positive) ranged from

71 to 90%, and the specificity (true negative) range was 83 - 100%²⁵.

Successful clinical tumour imaging with anti-CEA, was achieved only when blood pool background was subtracted by a computerized technique²⁵⁻²⁷. Simultaneous injection of ^{99m}Tc-HSA (human serum albumin) and ^{99m}Tc-pertechnetate permits computerized-subtraction of the blood pool and free radioiodide from the radiolabelled antibody distribution. Tumour:nontumour ratios of radioactivity were only 1.4 prior to background subtraction in one study but the tumour images were enhanced 2.5 times following computer-assisted processing of the images²⁵. The absolute requirement of this subtraction technique for successful tumour imaging has led some investigators to question the present suitability of anti-CEA for routine clinical use²⁷.

Radiolocalization studies have also been reported which use antibodies to human chorionic gonadotropin (HCG)²⁹ and alpha-fetoprotein (AFP)³⁰. As with anti-CEA, a slow blood clearance in these investigations did not permit tumour-specific localization significantly above background tissue. Therefore, computer-assisted background subtraction and special calculations were required to demonstrate tumour selectivity of the antibodies²⁹⁻³⁰.

High amounts of circulating antigen do not appear to prevent successful radioimmunodetection in the studies done to date^{25, 30}. Furthermore, no untoward or hypersensitivity reactions have been reported following the injection of

the xenogeneic immunoglobulins^{25, 30}.

The principal difficulties with the use of radioactively-labelled antibodies have been (1) the preparation of ultraspecific antibodies to tumours and (2) suppressing the background radioactivity caused by unbound antibody or antibody-antigen complexes in the circulation²¹.

Upon injection of an antigen macromolecule into an animal, many cross-reactive antibodies are synthesized, due to the availability of numerous antigenic sites on a macromolecule¹⁸. The resulting antibody preparation will not be homogeneous and will be able to detect and react with many more antigens than the specific desired tumour marker. Attempts to rectify this problem have involved (1) the isolation and purification of the specific antigenic determinant of the tumour marker and (2) the production of monoclonal antibodies.

Kohler and Milstein³¹ developed a method in which hybridomas were used to produce homogeneous and reproducible monoclonal antibodies which are reactive to only one antigenic determinant. Antibody-secreting plasma cells, specific for the antigen, are hybridized or fused to myeloma cells which provide the ability to grow continuously in culture as hybridoma cells³¹. Monoclonal antibodies have been developed towards various tumour markers and have been used in the radioimmuno-detection of the metastases of hepatoma, colorectal cancer and choriocarcinoma²⁷. Unfortunately the technique is relatively complicated,

sophisticated and labour intensive.

Another type of homogeneous protein which, like monoclonal antibodies, can specifically interact with cell surface residues is a class of proteins, called lectins.

B. LECTINS IN GENERAL

1. Definition and Origins

The word lectin comes from the Latin legere meaning to pick out or choose. Boyd and Shapleigh³² originally applied the term to a group of carbohydrate-binding plant seed (glyco) proteins which could distinguish among human blood groups. Sugar-binding proteins, which interact with and agglutinate plant and animal cells, have now been identified in a diverse range of organisms, including: bacteria, molds, and algae, plants, sponges, fish, snails, eels, crabs and even mammals³³. A definition which has now been adopted for the word lectin is "a sugar binding protein (or glycoprotein) of non-immune origin which agglutinates cells and/or precipitates glycoconjugates"^{33, 35}. Reviews of the properties, actions and uses have been recently published^{33, 36-39}.

2. Macromolecular Properties

Although crude saline extracts of lectin were initially used, many lectins currently employed are purified to homogeneity by affinity chromatography on

columns containing carbohydrate supports, as reviewed by Lis and Sharon⁴⁰.

Studies of those lectins obtained in purified form, have revealed a diverse range of physical and chemical characteristics³³. There is no structural feature common to all lectins, except that they are proteins³³. All lectins consist of subunits, although the number of subunits per molecule varies⁸. Some lectins require metal ions such as Ca^{+2} or Mn^{+2} for biological action³³. Molecular weights of lectins have ranged from 36,000 to 335,000³⁹.

Sometimes the lectins occur as a group of closely related proteins, called isolectins, which have very similar chemical and biological properties but differ in electrophoretic mobility⁴¹. Many of the lectins are glycoproteins, although several lectin proteins, such as peanut lectin, concanavalin A and wheat germ, lack covalently linked sugars³⁹.

3. Antibodies versus Lectins

Lectins are similar to antibodies in many respects³⁹. The combining site of the lectin interacts specifically with the carbohydrate-bearing structure, to induce agglutination or precipitation³³. This is similar to antibody-antigen reactions because it is very specific and also reversible. In addition, the interaction can be specifically inhibited by low molecular weight "haptens" which block the combining site.

The binding site of the lectin is usually smaller than that of an antibody. Simple monosaccharides can often inhibit lectin interactions, but the smallest fragment which will inhibit precipitation by antibodies is a disaccharide⁴².

Other major differences exist between antibodies and lectins. Antibodies are glycoproteins secreted by lymphocytes, as an elicited response to a foreign stimulus⁴². In contrast, the organisms in which lectins originate, are often not able to produce an immune response and the lectins are usually present as constituent proteins³⁹. Another difference pertains to the specificity range. While the specificity of lectins is restricted to simple and complex carbohydrates, antibodies can be formed to react specifically with other classes of organic compounds, including amino acids, proteins and nucleic acids³⁹. The antibodies that are formed are structurally similar. This is in contrast to the structural diversity observed for lectins isolated from various sources³⁹.

In spite of these differences, lectins have often been used as models of carbohydrate-specific antibodies and have been used to replace them in techniques such as blood grouping^{33, 43}. Lectins are readily available and are easily purified in gram quantities. In addition, the combining site of purified lectins, unlike that of most immune antibodies, is small and homogeneous³³. Lectins therefore have important application in the study of the

carbohydrate architecture of cell surfaces⁴⁴ .

4. Role in Nature

The physiological role or function of lectins in animals and plants is not fully understood⁴⁵ . In plants, lectins may be involved in sugar transport, storage or immobilization, and may function as protective agents by specific interaction with the polysaccharides of plant pathogens⁴⁵ . The presence of lectins in leguminous plants may be related to the plant's ability to fix nitrogen. It has also been speculated that plant lectins may facilitate seed germination and early cell division and differentiation⁴⁵ .

Some animal lectins possibly serve as recognition molecules to sequester glycoproteins. Others may play a role in the organization of cells into tissues during embryogenesis or be involved in cellular communication and/or adhesion⁴⁵ .

Although definite functions can not yet be assigned to any lectin, it appears the variety of intracellular and cell surface functions postulated for lectins involve specific interaction of the lectins with carbohydrate-containing substances⁴⁵ .

5. Binding Specificities

The overall carbohydrate specificity of a lectin is usually defined by the monosaccharides or oligosaccharides

that have the strongest inhibitory effect on the lectin-induced agglutination or precipitation reactions³⁵. The sugar specificity and origin of a number of commonly available lectins are listed in Table 2.

Lectins usually have stringent structural requirements for optimal binding³⁶. Often the C-3 and C-4 hydroxyl groups of sugars appear to play an important role in lectin binding³³. The generality that D-galactose binding lectins do not usually interact with D-glucose or D-mannose specific lectins and vice versa demonstrates the critical involvement of the C-3 hydroxyl moiety³³.

Although the binding site of most lectins accomodates a single glycosyl residue, some lectins have extended saccharide binding sites³³. The peanut lectin (Arachis hypogoea), has a binding site complimentary to a disaccharide and the affinity of wheat germ agglutinin (Triticum vulgare) for $\beta(1\rightarrow4)$ -linked D-GluNAc increases for the trisaccharide series of the same sugar residue³⁷.

The binding of lectins to complex oligosaccharides, glycoproteins and cells is a more complex phenomenon than that seen with simple sugars³⁸. Multivalent and secondary nonspecific interactions are superimposed on the primary, carbohydrate-specific binding. Therefore, the association constants for lectin-glycoprotein binding is usually several orders of magnitude higher than those found for monosaccharides³⁹.

TABLE 2//Lectins: Their Carbohydrate Binding Specificity and Origin

Genus and Species	Common Name	Origin	Binding Specificity	Reference
D-Mannose (D-Glucose) binding lectins				
<i>Canavalia ensiformis</i> (Con A)	Jack-bean	plant	D-Manul+	33,46,48,49
<i>Lens culinaris</i>	lentil	plant	D-Manul+	33,46,48,49
<i>Pisum sativum</i>	pea	plant	D-Manul+	33,46,48,49
<i>Vicia faba</i>	—	bacteria	D-Manul+	47
<i>E. coli</i> , <i>Ps. aeruginosa</i>	—	—	D-Manul+	46
N-Acetyl-D-Glucosamine-binding lectins				
—	bird & reptile livers	vertebrates	D-GlcNAcBl+	46,47
<i>Bandeiraea simplicifolia</i>	BS-II	plant	D-GlcNAcBl+	33,46,47
<i>Triticum vulgare</i> (WGA)	wheat germ	plant	(D-GlcNAcBl+4); chitin oligomers	33,46,48,49
<i>Solanum tuberosum</i>	potato	plant	(D-GlcNAcBl+4)	33,46,47
<i>Datura stramonium</i>	jimson weed	plant	(D-GlcNAcBl+4)	33,46
<i>Phytolacca americana</i>	pokeweed	plant	(D-GlcNAcBl+4) (complex)	47
L-Fucose-binding lectins				
<i>Anguilla anguilla</i>	eel	vertebrate	L-Fucal+	46
<i>Ulex europaeus</i> I	gorse seed	plant	L-Fucal+2DGalBl+4GlcNAcBl+	33,46,48,49
<i>Lotus tetragonolobus</i>	asparagus pea	plant	L-Fucal+2DGalBl+4-[L-Fucal-3]-DGlcNAcBl+	33,46,48,49
Sialic Acid				
<i>Limulus polyphemus</i>	horseshoe crab	invertebrate	sialic acid	46
N-Acetyl-D-Galactosamine-binding lectins				
—	lobster lectin II	invertebrate	D-GalNAcBl+	46
<i>Helix pomatia</i>	edible snail	invertebrate	D-GalNAcBl+	33,46
<i>Phaseolus lunatus</i>	lima bean	plant	D-GalNAcBl+	33,46,48
—	rabbit liver	vertebrate	D-GalNAcBl+	46
<i>Sesophora japonica</i>	Japanese poganda tree	plant	D-GalNAcBl+	33,46,48
<i>Glycine max</i>	soybean	plant	D-GalNAcBl+3-DGalBl+3DGlcNAcBl+	33,46,48,49
<i>Dolichos biflorus</i>	horse gram	plant	D-GalNAcBl+3(LFucal-2)DGalBl+	33,46,48,49
D-Galactose binding lectins				
<i>Dictyostelium discoideum</i>	slime mold	mold	D-GalBl+	46,47
<i>Ricinus communis</i>	castor bean	plant	D-GalBl+	46,47
<i>Cerianthus membranaceus</i>	sea anemones	invertebrate	D-GalBl+	46,50
—	liver, muscle, milk, lung, heart	vertebrate	D-GalBl+	46,47,50,51
<i>Electrophorus electricus</i>	electric eel	vertebrate	D-GalBl+	46,47,50
<i>Pseudomonas aeruginosa</i>	chick embryo mushroom	bacteria	D-GalBl+3 (complex)	51
<i>Agaricus bisporus</i>	sponge	plant	D-GalBl+3 (complex)	33,46,50
<i>Bauhinia purpurea</i>	chick embryos	plant	D-GalBl+3	46,47
<i>Geodia cydonium</i>	sponge	sponge	D-GalBl+4	46,47
<i>Axinella</i>	sponge	vertebrate	D-GalBl+	52
<i>Tridacna maxima</i>	sponge	invertebrate	D-GalBl+6	46,50
<i>Arachis hypogaea</i>	peanuts	plant	D-GalBl+3DGlcNAcBl+	46,50

Key: Fuc=fucose, Gal=galactose, GalNAc=N-acetyl-D-galactosamine, GlcNAc=N-acetyl-D-glucosamine, Man=mannose

In addition, lectins which have a similar specificity towards monosaccharides, many show large variability in their binding specificity to complex heterosaccharides³⁹.

6. Biological Actions and Uses of Lectins

Lectins have been very useful for the investigation of various cellular phenomena due to the specific interaction with specific carbohydrate determinants on the cell surfaces^{37, 38, 43, 44}. Following labelling with fluorescent tags, ferritin or radiotracers, lectins have been useful as cell surface probes. The nature, distribution and number of lectin receptors were then investigated by either autoradiography or fluorescence microscopy or at an ultrastructural level using electron microscopy^{37, 53}.

Certain lectins, such as the pokeweed mitogen, concanavalin A and the red kidney bean lectin (PHA) are mitogenic. These lectins stimulate lymphocytes to grow and divide, regardless of the antigenic specificity of the lymphocytes and are, therefore, an important tool in the field of immunology³⁹.

The specificity of most blood groups is determined by carbohydrate residues, so that lectins have been very useful in clinical blood typing and structural studies of blood group substances⁴³.

The interaction of lectins with many cells is most easily detected by cell agglutination^{37, 38, 44}. The

agglutination reaction is a very complex phenomenon in which many factors play a role, as reviewed by Nicolson^{37, 44}. Lectins may bind to cell-surface sugar residues without inducing agglutination of the cells³⁹. In order for the agglutination to occur, the lectin must be able to form multiple cross-bridges between opposing cells³⁹.

Lectin studies have revealed that the ease of cell agglutination is significantly different between normal and malignant cells^{37, 49} between embryonal and adult cells⁴⁴ and also between mitotic and quiescent cells⁴⁴. Lectins have therefore been useful tools to study cell surface changes which occur during malignant transformation^{37, 44, 49} and embryonic cell differentiation^{44, 52}. The increased agglutinability of most transformed cells is an interesting phenomenon in view of the fact that they generally possess a similar number of lectin receptors as their untransformed counterparts^{37, 44, 49}. Differences in agglutinability are probably due to an increased mobility of lectin receptors on transformed cells³⁷. Fluorescent microscopy revealed that receptor sites, following lectin binding, were no longer randomly distributed in tumour cells but were aggregated into clusters. This enables lectins to readily form multiple cross-bridges between cells, thus facilitating agglutination. When the receptor sites are fixed in their initial random dispersion, by glutaraldehyde or formaldehyde treatment, the transformed cells are no longer preferentially agglutinated^{37, 44, 49}.

An important application of lectins has been the separation and purification of glycoproteins and cells^{37, 38, 53}. Immobilized lectin columns have been applied extensively as an affinity purification technique and used for the fractionation of animal cell subpopulations⁵³.

7. In Vivo Studies With Lectins

Although most studies involving lectins have been in vitro, lectins have been used in vivo as carriers for the delivery of chemotherapeutic agents to tumours⁵⁴⁻⁵⁶. This follows attempts to preferentially localize chemotherapeutic agents in tumours using antibodies as carriers, as reviewed by Ghose and Blair⁵⁷.

The lectin which has often been utilized as a carrier of antitumour drugs, has been concanavalin A⁵⁴⁻⁵⁶. Concanavalin A has its own antitumour activity. It selectively agglutinates and kills some tumour cells in vitro and can increase the life span of tumour-bearing mice⁵⁸. Injection of a con A-chemotherapeutic drug complex was found to be more effective in prolonging survival of tumour bearing mice than injection of either the lectin or anti-tumour drug alone⁵⁴⁻⁵⁶. Since con A is a powerful mitogen and may induce haemorrhagic Arthus-like reactions, hydrocortisone was sometimes given concurrently with the con A complexes⁵⁵.

The success of lectins as carriers of chemotherapeutic agents for tumour localizing and cancer treatment encourages

the possible application of radiolabelled lectins as diagnostic tumour localizing agents. Ideally, the lectin should not be toxic to normal cells, not mitogenic and should interact specifically with sugar residues which are primarily associated with malignancy.

C. PEANUT (ARACHIS HYPOGOEA) LECTIN (PNA)

The peanut (Arachis hypogoea) lectin is a readily available protein, which binds preferentially to oligosaccharides containing the terminal sequence β -D-galactosyl-(1+3)- α -N-acetyl-D-galactosamine (β DGal(1+3)GalNAc)^{59, 60, 61}. This disaccharide is reported to be the immunodeterminant group of the Thomsen-Friedenreich antigen (T antigen)⁶². The T antigen is present in a number of membrane and soluble glycoproteins but it is normally masked by N-acetyl-neuraminic (sialic) acid residues⁶³. Therefore, the peanut lectin will only bind to the glycoproteins following exposure of the T-antigen by neuraminidase treatment.

The affinity of the peanut lectin (PNA) for the T antigen, allows this protein to agglutinate neuraminidase-treated red blood cells. It was therefore designated an "anti-T agglutinin" since it gave the same immunological reaction as the anti-T antibody of mammalian sera⁶⁴.

A special importance and clinical relevance became associated with the T-antigen, following its discovery in

the cell membranes of human carcinomata⁶⁵. The tumour-associated T specificity has been found in the unmasked, reactive form in adenocarcinoma of the breast, gastrointestinal, and respiratory tract⁶⁶.

The expression of the T antigen on malignant tissue, and not on corresponding benign or normal tissue, may represent incomplete synthesis or accelerated degradation⁶⁵.

Studies, using peanut lectin on sections of human breast tissue, have demonstrated a pattern of T antigen expression which differs markedly between benign and malignant tissues⁶⁷.

The above observations indicate that radiolabelled peanut lectin may therefore be useful for the in vivo localization of tumours expressing the T antigen.

1. Macromolecular Properties of PNA

Affinity-purified peanut lectin (PNA) has been determined to be homogenous by methods such as disc-gel electrophoresis, gel filtration, ultracentrifugation and sedimentation analysis. Lotan and coworkers⁵⁹ determined that the intact peanut lectin protein exhibited a molecular weight of $110,000 \pm 10,000$, as determined by gel filtration and sedimentation velocity. This value was in close agreement with a value of 106,500 determined by Terao and coworkers⁶⁸ employing sedimentation equilibrium centrifugation. When Fish et al.⁶⁹ analyzed the lectin by sedimentation equilibrium and also gel filtration,

under a wide range of pH and concentration ranges, they obtained a lower molecular weight value of $98,000 \pm 3,000$. Possible reasons for this discrepancy could be (1) method of extraction used by Lotan and Terao possibly leading to slight aggregation⁶⁹, (2) higher protein concentrations used by Lotan and Terao (2.5 - 10 mg/ml) may result in a lower S^{0}_{20w} ⁶⁹, which results in a higher estimate of molecular weight, and (3) isolectin variation⁷⁰.

The peanut lectin has recently been crystallized, for X-ray studies, into an asymmetric orthorhombic crystal with 57% of the volume being solvent-occupied⁷¹. It is a relatively acidic (pI = 5.95)⁶⁸ hydrophilic, and compactly folded globular protein⁶⁹. The lectin is believed to be composed of four identical monomers of 24,500-27,000 dalton molecular weight, which are non-covalently linked^{59, 68, 69}. There are 4 binding sites per molecule (revealed by equilibrium dialysis) and metal analysis by atomic absorption analysis indicates that each PNA subunit contains one Ca^{+2} and Mg^{+2}/Zn^{+2} atom (0.78 mole Mg^{+2} per subunit and 0.11 mole Zn^{+2} /subunit)⁷².

The quaternary structure of PNA is pH dependent⁶⁹. As the pH is gradually decreased from 4.75 to 3.0, the molecule reversibly dissociates from a tetramer into a dimer. The 48,000 dalton species which exists at pH 3 lacks sugar binding ability⁶⁹. Denaturing conditions or detergents (sodium dodecylsulfate) dissociate the intact molecule into its 4 subunits, which have identical molecular

weights^{59, 68, 69} and identical sequencing for the last five NH₂-terminal amino acids⁵⁹.

The amino acid composition reported by Lotan and coworkers⁵⁹ showed a high content of acidic and hydroxylic amino acids, relatively little methionine, tryptophan and histidine and the complete absence of cysteine. These results were at variance with the results of Terao and coworkers⁶⁸, who reported considerably less serine, threonine (which Lotan et al.⁵⁹ determined only by extrapolation), tryptophan and arginine. Terao⁶⁸ also did not find any methionine but reported 16.6 moles of cysteine per mole of lectin. Neither group found covalently-bound carbohydrate^{59, 68}.

The first 40 amino acids of peanut lectin N-terminus have been sequenced⁷³. It has been found that there is considerable homology between the first 25⁷⁴ and the first 40⁷³ residues of PNA and several other lectins isolated from legumes (e.g. soybean, lentil and pea). This may suggest a common ancestry of the genes coding for these lectins^{73, 74}.

2. Isolation and Purification of PNA

Isolation of lectins generally begins with a saline or buffer extraction of the finely-ground seed meal. The anti-T activity was initially discovered in crude saline extracts such as these⁶⁴ and some investigators still use them, as such, without further purification^{75, 76}.

As much as 40 per cent of the net weight of peanuts is composed of lipid components, some of which may be contained in the crude saline extracts. Therefore, a pre-extraction step with organic solvents (such as ether or acetone) is often employed to remove lipidic or other interfering substances^{59, 68, 77 80}.

The proteins, from the defatted preparation, are then usually salted out with neutral salts such as 40-75% ammonium sulfate^{59 68 77 79}. The precipitate is then collected by centrifugation before being redissolved for dialysis and/or ultracentrifugation^{59, 68, 77 79}.

The clear supernatant will contain a number of proteins besides the lectin, such as the large molecular weight peanut globulins, known as arachin (MW^v380,000) and conarachin^{81, 82}. The lectin can be isolated from this protein mixture by conventional protein-purification techniques, affinity chromatography or a combination thereof³³.

A novel method, for the purification of anti-T from peanuts, was recently described by Merry and coworkers⁸⁰. They used the reagent Rivanol^R (2-ethoxy-6,9,-diamino-acridine lactate), which is known to precipitate all proteins except IgG when added to serum⁸⁰. Following the addition of Rivanol to peanut saline extracts, the majority of the peanut proteins were selectively precipitated to leave the anti-T in solution. The method was rapid and simple and produced a serologically-active

purified form comparable to that prepared by affinity chromatography⁸⁰.

Virtually most of the other contemporary lectin-purification schemes employ affinity chromatography techniques, which exploit the specific sugar-binding capacity of the lectin⁴⁰. A carbohydrate ligand with which the lectin interacts is insolubilized in some manner, so as to allow for specific adsorption of the lectin. The lectin can then be displaced by elution with a sugar which is competitive for the same binding site^{33, 40}. A large variety of affinity adsorbents have been used for peanut lectin, as presented in Table 3.

The biospecific adsorbents of lectins may be divided into 3 major types: (1) native or modified polysaccharides (examples 1-3, Table 3), (2) matrix-bound glycoproteins or glycopeptides (examples 4,5), and (3) matrix-bound mono- and disaccharides (examples 6-10). They all have in common terminally exposed galactose residues to which the peanut lectin can be specifically and reversibly bound.

The immunodeterminant disaccharide (β -D-Gal(1 \rightarrow 3)- α -D-GalNAc) of the T antigen has recently been synthesized into an immunoadsorbent form⁸². The availability of this immunoadsorbent, for the purification of very specific preparations of anti-T antibodies and peanut lectin, should prove very useful for T antigen studies.

TABLE 3

Affinity Purification Methods for Peanut Agglutinin

Matrix	Ligand	Coupling Method	Reference
1. acid-treated sepharose 6B	-	-	83,84
2. insolubilized guar gum	-	-	78,85
3. cross-linked arabinogalactan	-	-	71,77
4. cross-linked desialylated erythrocyte stroma	-	-	40
5. sepharose	desialyated fetuin	CNBr activation	79
6. sepharose	ϵ -aminocaproyl-N-galactosylamide	CNBr activation	59,85,87
7. divinyl-sulfone activated sepharose	lactose	high pH	69,70,90
8. aminoethyl polyacrylamide	lactose	cyanoborohydride reduction	88
9. acrylamide	alkyl glycoside of galactose	co-polymerization	78
10. chromosorb	β DGal(1+3) α GalNAc	azide coupling	89

3. Isolectins: Multiple Molecular Forms of PNA

The presence of several very similar lectins has been observed in a number of seed extracts. These multiple molecular forms of agglutinins, or isolectins, differ in their electrophoretic mobilities⁴¹. Individual genotypes in the species of the lectin origin may have same spectrum of isolectins as each other or there may be variations in the number and types of isolectins available in each genotype⁷⁰.

Lectins extracted from peanuts have been purified by affinity chromatography and appear homogeneous by most protein separation techniques. Newman⁸⁷ resolved affinity-purified peanut lectin into several isolectins, with very similar isoelectric points, by polyacrylamide gel electrophoresis and isoelectric focusing. All the isolectins were composed of subunits with the same terminal amino acid (alanine) and molecular weight (28,000) and they all agglutinated neuraminidase-treated erythrocytes to the same extent⁸⁷.

Affinity-purified lectins exhibiting the binding, subunit composition and electrophoretic properties characteristic of peanut lectin^{59, 90} were identified from two separate Arachis hypogoea genotypes and could be separated by polyacrylamide gel isoelectric into 6 and 7 agglutinating isolectins each⁹⁰.

The isoelectric points, (ranging from 5.7 to 6.7), and the occurrence in various Arachis hypogoea genotypes, were

the only characteristics found to vary between the isolectins. None of the isolectins were removed by further affinity chromatography and all the isolectins agglutinated neuraminidase-treated red blood cells. Each isolectin was composed of 27,000 molecular weight subunits and antibodies raised to one genotype were immunologically cross-reactive to the other strains of Arachis hypogoea⁹⁰.

Recently, a large number of peanut genotypes and related Arachis species were immunologically screened to determine the presence of PNA and the isolectin profiles in various genotypes⁷⁰. Peanut lectin, as determined by anti-PNA rabbit antibody, was detected in 4556 genotypes of Arachis hypogoea and 65 genotypes of related species of Arachis⁷⁰. The lectin was lacking in only 4 wild Arachis strains (<0.1% of all the strains which were tested). Polyacrylamide gel electrophoresis/isolectric focusing resolved Arachis hypogoea lectins into 3 related isolectin profiles, each composed of 6 to 8 isolectins⁷⁰. In spite of variations in isolectin profiles, the specific hemagglutinating activities and electrophoretic properties were quite uniform amongst genotypes⁷⁰. It still needs to be determined if biological attributes, such as binding constants and mitogenic ability, co-vary with isolectin profile variations.

4. Protein Stability of PNA

Peanut lectin is a protein and, as such, may be adversely affected by extreme changes in pH or temperature.

In the native seed form, most lectins have been found to be very resistant to deterioration on storage, so that active lectins could be extracted 25 to 30 years later⁴⁴. Peanut lectin has shown some resistance to dry heat, as peanut lectin activity has still been detected in dry-roasted peanuts³².

In crude saline extracts of peanuts, a stable hemagglutination activity was reported over 6 months of storage at -20°C ⁷⁵. An affinity-purified peanut lectin was reconstituted after lyophilization to a concentration of 1 mg/ml and was reported to remain stable for 2 years, at 4°C in the presence of bovine serum albumin⁹³. In addition, the different durations of storage (at -20°C) of affinity purified PNA, and alternating cycles of freezing and thawing of isolectin solutions has been reported to not diminish biological activity of samples or to affect the isolectin composition⁹⁰.

The protein structure and conformation of peanut lectin, as revealed by UV spectra, was found to remain constant over: concentration ranges of 0.5 - 2 mg/ml, temperature ranges of 0.8 - 52°C and pH ranges of 3 - 10.75⁸⁵. In general, the peanut lectin appears to be a relatively "resilient" protein.

Although not reported by other investigators, Decastel and coworkers⁸⁵ found the peanut lectin was insoluble at low (eg. 4°C) temperatures. The cryoinsolubility was most likely to occur at high concentrations, low temperatures,

and in the pH range of 5 - 9. With a number of spectrophotometric methods (absorption, CD, fluorescence), it was determined that the observed cryoinsolubility was not due to conformational changes in PNA during the low temperature exposure. In addition, ultracentrifugation did not reveal evidence of polymerization of the PNA protein⁸⁵.

The cryoinsolubility of PNA was marked by a highly concentration-dependant lag time and a critical concentration of 0.8 mg/ml, below which the protein was completely soluble. Such features are characteristic of protein associations requiring a nucleation event⁸⁵.

It was concluded that the cryoinsolubility of PNA was probably due to the individual solubility properties of the lectin itself. At low temperatures, intermolecular association, by either hydrogen bonding or Van der Waals forces, may be preferred over interaction with the solvent⁸⁵.

It was found that the cryoinsolubility was partially reversible and totally inhibited in the presence of galactosides, the specific ligands of PNA⁸⁵. Their efficacy, as inhibitors of cryoinsolubility, was related to their binding affinity for the lectin. The disaccharide, β -D-Gal(1 \rightarrow 3)- α -D-GalNAc, was best able to inhibit the low temperature precipitation. It was, therefore, postulated that the residues responsible for aggregation are either localized in the sugar-binding site or in the vicinity of it and become masked, or locally reorganized during the binding of the saccharide⁸⁵.

General protein extracts of peanuts, containing arachin and conarachin, have been reported to also have this property of cryoinsolubility⁹⁴. Perhaps the unique extraction methods (no organic "defatting" step) and guar gum purification method of Decastel and coworkers⁸⁵ has isolated a peanut protein fraction different from that isolated by others.

5. Toxicity of PNA

Some lectins are highly toxic to mammalian cells, as discussed in a review by Nicolson⁴⁴. Two very cytotoxic lectins are abrin, from Abrus precatorius, and ricin from the castor bean (Ricinus communis). These lectins specifically bind to sugars on the cell surfaces and following cell uptake, they strongly inhibit protein synthesis⁹⁵. Oral or parenteral administration of abrin or ricin in high doses may be extremely dangerous or even lethal, for these plant toxins are approximately as toxic as the diphtheria toxin⁴⁴.

Other cytotoxic lectins, such as Concanavalin A, wheat germ agglutinin, and phytohemagglutinin, are 1000X less toxic than ricin and abrin⁹⁵. At very high concentrations (1-10 ug/ml), these lectins probably exert their toxicity via extensive specific binding to a high number of oligosaccharide sites on cell surfaces⁹⁵. The peanut lectin was not found to be cytotoxic to the cells with which it specifically bound (such as neuraminidase-treatment rat

lymphocytes) at in vitro concentrations as high as 600 $\mu\text{g/ml}$ ⁹⁶. Extensive toxicity studies have not been carried out on the peanut lectin.

Peanuts have been shown to have a beneficial effect for hemophilia, possibly due to an inhibition of fibrinolysis⁹⁷. Roasted peanuts, peanut butter, raw peanuts, peanut meal or flour and an alcoholic extract of peanuts have all been ingested by humans in large doses for their hemostatic action and no adverse effects were reported⁹⁸. Peanut meal or flour, prepared by defatting peanuts with hexane, was used in oral daily doses of 40 to 220 grams. This corresponds to an oral dose of 60 to 330 mg of peanut lectin, based on extraction and purification yields of Lotan⁵⁹. This daily regimen was used in several patients for longer than 6 months, without mention of toxic symptoms⁹⁷. The hemostatic factor in the flour is probably not protein in nature because it was removed by extraction with 90% ethanol⁹⁸.

Intravenous administration of the peanut lectin may result in the organism activating the humoral and cell-mediated sectors of the lymphoid system⁹⁹. Since the peanut lectin is a complex protein macromolecule of molecular weight $>10,000$, it will be recognized as a foreign antigen by the immunologically-competent cells of the organism⁹⁹.

The immunological reactions which usually protect the organism, may occasionally harm the organism, through

hypersensitivity or allergic reactions. Depending upon the manner (dose and route) in which the antigen is presented to the organism and the genetic constitution of an individual, there may be IgE antibody production induced in the organism⁹⁹. Reagenic IgE and other immunoglobulins are attached to mast cells or basophils, so that mediators, such as histamine, will be released upon antigen interaction⁹⁹. These mediators act upon tissues to cause vasodilation, smooth muscle contraction, and mucous secretion⁹⁹. Consequently, the classic manifestations of the immediate hypersensitivity reactions, such as anaphylaxis may ensue⁹⁹.

Acute anaphylaxis in man is a rare, though serious, reaction with possible fatal consequences. Certain people have a genetic predisposition to become easily sensitized and allergic. These people are called atopic. The occurrence and intensity of anaphylactic reactions in an atopic individual depends on factors such as: the nature and quantity of antibodies present, the target cell reactivity, the response of anatomic structures sensitive to the mediators and control from the autonomic nervous system⁹⁹.

A more frequent form of anaphylaxis is found in the form of a localized phenomenon, following antigen contact with specific sensitized organs or tissues, such as epithelial barriers. Local anaphylactic reactions occur in the skin (eczema and urticaria), respiratory tract

(pollen allergies and bronchial asthma), and the digestive tract (food allergies)⁹⁹.

Peanuts are a frequent source of food allergies. The hypersensitivity reactions can range from mild abdominal discomfort to severe life-threatening anaphylactic shock^{100,101}. Double blind food challenges with peanuts in 81 children with a food-allergy history revealed that 17 (21%) of these patients were allergic to peanuts¹⁰⁰. Peanuts showed the highest positive response of the foods tested in that study. Another study indicated that 16 of 84 allergic children had elevated IgE levels and the IgE was reactive with peanut extracts¹⁰¹.

Recently, investigators have attempted to isolate and identify the allergen in peanuts responsible for allergic reactions¹⁰². A protein fraction (Peanut-I), isolated by ion exchange chromatography from crude peanut extracts, was found to be the major allergen but could not account for all of the allergenic activity of peanut extracts¹⁰².

The major allergen fraction, Peanut-I, probably does not include the peanut lectin, since it contained 8.7% carbohydrate, and gel chromatography revealed that it had a molecular weight of 180,000 Daltons. Studies will need to be done on the allergic potential of peanut lectin, especially in those patients with a history of food allergy.

6. Mitogenicity

One of the most intriguing properties that some lectins possess, is the ability to trigger quiescent, non-dividing lymphocytes into a state of growth and proliferation. Mitogenic lectins are considered polyclonal activators, because they can stimulate a large proportion (often as much as 30-60%) of the cells, regardless of the antigenic specificity of the lymphocytes³⁹. The gross changes, in size and shape and biochemical events, observed in the laboratory for lectin-stimulated lymphocytes appear to resemble the specific antigen-induced immune reactions in the living animal^{44, 103}. Therefore, various mitogenic lectins, such as concanavalin A and the phytohemagglutinin of the red kidney bean, have been used extensively to investigate the mechanism of immune response⁴⁴.

Like agglutination, mitogenic stimulation follows the specific binding of a lectin to the saccharide units of glycoproteins or glycolipids, on the cell surface³³. This binding, however, is not by itself sufficient to induce cell activation and a mitogenic response¹⁰³. There are many lectins that bind to lymphocytes but do not stimulate them¹⁰⁴.

The mitogenic ability of peanut lectin is a controversial question. Terao and coworkers⁶⁸ could not demonstrate mitogenicity of peanut lectin towards either normal or neuraminidase-treated human lymphocytes at

in vitro concentrations of up to 250 µg/ml. On the other hand, Novogrodsky and coworkers⁹⁶ observed the mitogenic response (of increased DNA synthesis) in neuraminidase-treated rat and human lymphocytes, following the binding of this lectin. Untreated lymphocytes did not respond; nor did normal or neuraminidase-treated lymphocytes of the mouse or guinea pig. Both groups of authors reported their preparations to be homogeneous^{68, 96}.

The source of discrepancy may be due to variations in isolectins of the peanut lectin utilized in the mitogenic testing. The types of isolectins obtained in a peanut lectin preparation are dependant on the peanut variety extracted and purification method used⁷⁰. Slight variations in amino acid content change the molecular charges in the isolectins and allow electrophoretic separation of an affinity-purified lectin into 6-7 isolectin bands⁷⁰. Although these minor amino acid changes do not affect the hemagglutination activity of the PNA, they may be the factors responsible for the different mitogenic properties^{87, 90}. No correlation between mitogenic and hemagglutination ability has been found between various isolectins⁸⁷.

Another source of variation in mitogenic ability, may be due to the presence of aggregated or polymerized peanut lectin in the preparation. When peanut lectin was cross-linked or polymerized by glutaraldehyde treatment, it acquired mitogenicity to neuraminidase-treated mouse

lymphocytes that the lectin lacked in its native, monomer form¹⁰⁴. It was found that lectins which induce a mitogenic response, show a positive cooperativity of binding, meaning that the binding constant increases as more PNA occupies the cell surface receptors¹⁰⁴. This implies that the membrane receptors possibly undergo conformational changes or redistribution to increase the binding constant. It has been postulated that the "signal" for mitogenicity is mediated by a clustering of the receptors in the fluid membrane¹⁰³. When a lectin is aggregated into a multivalent form, this allows formation of an initial nucleus of cross-linked receptors¹⁰⁴. Nucleation then provides the "triggering signal" for cross-linking and clustering of the lectin-receptor complexes in the fluid membrane, leading to a mitogenic response^{103, 104}.

In one study, the presence of particulate erythrocyte fragments was found to enhance the mitogenic response of peanut lectin up to 100 fold¹⁰⁵. Perhaps these erythrocyte fragments had exposed PNA receptors, leading to PNA binding and aggregation and thereby, the supermitotic state of multivalent lectins.

Much more needs to be learned about the mechanism of lectin-induced mitogenicity and the characteristics of lymphocyte populations, before the relationship of peanut lectin and mitogenicity is fully understood.

7. Binding Specificity of Peanut Lectin

It is particularly important to know the sugar-binding specificities of lectins to use them in the detection and purification of cell surface carbohydrate and to study the biological activities of the lectins themselves.

Usually, lectin-saccharide specificities have been determined by making comparisons of the inhibition of hemagglutination or glycoprotein precipitation by various mono- and oligosaccharides³³. The binding preferences of lectins have also been determined by more direct measurements, such as equilibrium dialysis⁷², ultraviolet difference spectroscopy¹⁰⁶ and carbon-13 nuclear magnetic resonance (NMR) studies^{107, 108}.

a) Saccharide Inhibition of Hemagglutination or Precipitation

Early studies by Bird⁶⁴, using crude saline extracts of peanuts, indicated that galactose or lactose could weakly inhibit the hemagglutination of neuraminidase-treated red blood cells. It was therefore suggested that β -glycosidically-linked D-galactosyl residues might be an important part of the T antigen to which the lectin bound^{61, 64}.

Uhlenbruck and coworkers⁶¹ found particularly strong hemagglutination inhibition by 2-acetamido-2-deoxy-3-O- β -D-galactopyranosyl-D-galactose (β DGal(1 \rightarrow 3) α DGal-NAc) and, by glycoproteins and gangliosides carrying this disaccharide in a non-reducing (terminal)

position. Later studies confirmed these findings^{59, 60, 109}.

Various immunochemical investigations of the specificity of peanut agglutinin have been conducted by hapten-inhibition of hemagglutination and precipitation and are summarized in Table 4.

It is interesting to note that the purified peanut lectin was not inhibited by terminal N-acetyl-D-galactosamine, in contrast to most other D-galactose-specific lectins and agglutinins^{59, 60, 68}. In addition, many other D-galactose-binding lectins fail to accommodate more than a single glycosyl residue³³. On the other hand, the peanut lectin combining-site interacts, in a highly specific way, with the T determinant disaccharide (β DGal(1 \rightarrow 3)GalNAc) with an affinity 50 times greater than the galactosyl monosaccharide^{59, 60}. This suggests an extended combining site for the peanut lectin⁶⁰.

It is possible to specify the saccharide structural features required for combining with and blocking the peanut lectin binding site, if it is assumed that the binding inhibition activity of the saccharides, reflects the affinity of the peanut lectin for the particular sugars. Sugars which are most capable of inhibiting the binding of peanut lectin to neuraminidase-treated red blood cells, probably fit the specific binding site of the lectin in a much more complementary fashion than those sugars

TABLE 4

Inhibitory Effect of Various Sugars on Hemagglutinating and Glycoprotein Precipitating Activities of Peanut Agglutinin

Inhibitor	Relative Potency ¹ In Inhibition of:		
	Hemagglutination		Precipitation
	Lotan ²	Terao ³	Pereira ⁴
<u>Monosaccharides</u>			
1. D-Gal	1.0	1.0	1.0
2. D-Xyl	<0.4	-	-
3. L-Fuc	<0.4	<0.25	-
4. D-Fuc	<0.4	-	0.6
5. D-Glu, DGlunH ₂ or GluNac	<0.4	<0.25	-
6. DGalNH ₂	1.43	4.25	2.2
7. DGalNac	<0.4	<0.25	-
8. 6-0-methyl α DGal	-	-	1.0
9. methyl α DGal	2.5	-	2.2
10. p-Nitrophenyl α DGal	1.66	4.25	2.2
11. methyl β DGal	1.25	-	1.5
12. p-Nitrophenyl β DGal	1.25	2.0	1.5
<u>Di- and oligosaccharides and glycoproteins</u>			
13. DGal β 1+4DGlc (lactose)	1.66	2.0	2.2
14. DGal β 1+6DGlc	-	-	2.2
15. DGal β 1+4DGlcNac	1.66	-	4.0
16. DGal β 1+3DGlcNac	-	-	0.6
17. DGal β 1+3GalNac (T Ag)	50.0	-	54.5
18. DGal α 1+3D-Gal-ol-Nac ⁵	-	-	2.2
19. DGal α 1+3D-Gal	-	-	0.9
20. DGal α 1+6DGlc	0.8	-	0.6
21. DGalNac α 1+3Gal	-	-	< .5
22. Ch-3 (MN glycoprotein fragment)	-	20.0	-
23. asialo-Ch3	-	2,500 ⁵	-
24. MN glycoprotein	-	2.5	-
25. asialo MN glycoprotein	-	5,000	-

¹ Galactose assigned an inhibitory potency (ability to inhibit specific PNA binding) of 1 and all other sugars are calculated relative to galactose.

² The values are based on concentration of sugars needed for 50% inhibition of hemagglutination of neuraminidase-treated human type-B erythrocytes from Lotan *et al.*⁵⁹

³ The values are based on concentration of sugars needed to completely inhibit 4 times the hemagglutination dose to desialized human type AB erythrocytes. From Terao *et al.*⁶⁸

⁴ The values are based on concentration of sugars needed for 50% inhibition of precipitation of blood group glycoprotein. From Pereira *et al.*⁶⁰

⁵ Reduced T antigen determinant.

which do not inhibit binding. A number of important features of peanut lectin-ligands have thus been determined by the hemagglutination and precipitation inhibition studies of Lotan⁵⁹, Terao⁶⁸ and Pereira⁶⁰ and their coworkers. A brief description follows with the salient saccharide-features required for peanut lectin binding and the results of inhibition studies which support the importance of these features.

I. Hydrogen-bonding substituent on C-2 of terminal residue

→GalNH₂ is most effective while GalNAc is inactive for hemagglutination inhibition.

II. Unsubstituted 5-hydroxy-methyl group of terminal residue

→L-arabinose and fucose, which are lacking the hydroxymethyl groups, are inactive inhibitors.

→Saccharide derivatives (such as 6-O-methyl-galactose, D-galactose-6-sulfate, and D-galacturonic acid) which have masked hydroxyl groups, are also inactive inhibitors.

III. C-4 configuration of terminal residue similar to Galactose

→Stereoisomers with a conformation unlike Galactose at C-4 (such as D-glucose, D-Glu-Nac, D-mannose, L-fucose and maltose) are all inactive inhibitors.

IV. Pyranose form of terminal Galactose

→Methyl-galactose, which is fixed in a six membered ring, is a more effective inhibitor

than galactose, which is an equilibrium mixture of pyranose, furanose and an open chair form.

V. Pyranose form of penultimate GalNAc

→The reduced disaccharide is about 1/25th as active an inhibitor as the corresponding unreduced disaccharide.

VI. Anomeric carbon atom of terminal residue

→Discrepancies exist on the configuration of the anomeric substituent and vary with the size and electronegativity of the substituent.

→Large oligosaccharides which contain terminal

→ α -linked D-Gal are all weak inhibitors.

→ α -glycosides of D-Gal are slightly better inhibitors than β -anomers.

The combining site of the peanut lectin is probably in the form of a partial cavity, which only allows access to residues of a certain size limit⁶⁰. It has been found that the addition of various substituents onto the D-Gal of active inhibitors led to complete loss of inhibitory activity⁶⁰. The lactose disaccharide was extended to produce a longer sugar chain, and it was found that oligosaccharides which terminated in lactose were not anymore avid for peanut lectin-binding than lactose itself⁶⁰. It is possible, therefore, that the peanut lectin binding site is complementary for 2 sugar residues and does not interact specifically with sugars past the terminal

disaccharide⁶⁰. A hypothetical structure of the sugar-binding site of peanut lectin¹⁰³, with the complementary disaccharide β -D-Gal(1 \rightarrow 3) α -D-GalNAc, is schematically represented in Figure 1.

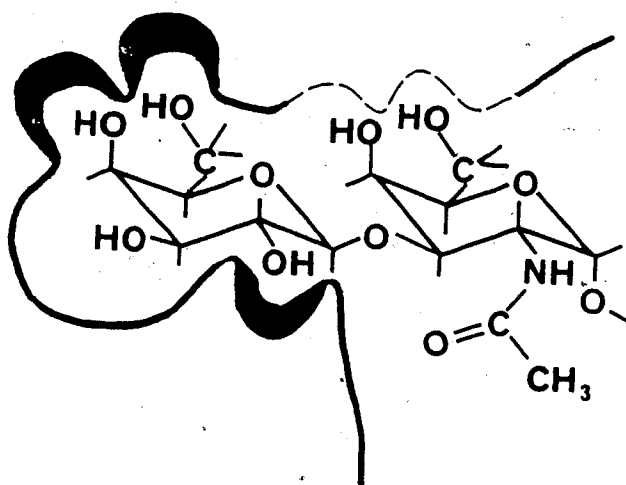


Fig. 1 Hypothetical sugar-binding site of peanut lectin

The interaction of lectins with polysaccharides, glycoproteins and glycolipids is much more complex than that with simple sugars³³. Glycoproteins and cell-surface carbohydrates usually have higher affinity constants for lectins than those observed for simple sugars³³. This is possibly due to the opportunity for multivalent interactions (all lectins have two or more binding sites³³) with the complex saccharides. Furthermore, nonspecific interactions

and steric influences must also be considered³³.

A chymotrypsin fragment of the MN glycoprotein, Ch-3, was shown to contain the disaccharide which is most complementary for the peanut lectin⁶⁸. Since the disaccharide is normally masked by sialic acid residues in this sialoglycoprotein, it does not normally interact with A hypogoea hemagglutinin unless the complementary disaccharide has been previously unmasked by neuraminidase-treatment⁶⁸. The inhibitory ability of this protein, in its native and asialo (neuraminidase-treated) form, are presented in Table 4.

The MN glycoprotein of erythrocytes, contains one of the most potent inhibitors of peanut lectin although it is in masked form. It is therefore necessary to remove terminal sialic acid residues from the intact MN glycoprotein, in order to reveal this very potent inhibitor⁶⁸. Glycoproteins that contain unsubstituted β DGal(1 \rightarrow 3) α DGalNAc sequences, such as asialoglycophorin, asialofetuin antifreeze glycoprotein and other T antigen expressing glycoproteins, are all precipitated readily by this lectin^{59, 68, 110}.

b) NMR and UV difference spectroscopy studies

The determination of lectin-binding specificity by inhibition of hemagglutination or precipitation has certain limitations. Hapten inhibition techniques assess relative inhibitor power and merely reflect the ratio of binding constants of different ligands. The

details of the binding process can be much better understood through thermodynamic and kinetic data, obtained by sophisticated techniques such as UV difference spectroscopy^{106, 107} and nuclear magnetic resonance studies^{107, 108}.

Like other plant lectins, PNA exhibits a near-UV circular dichroic spectrum which undergoes saccharide-induced transitions in the presence of those saccharides for which the lectin is specific⁶⁹. The environment of tyrosyl residues (represented by UV peaks near 280 nm), in or near the binding site of PNA, are altered upon specific interaction with sugars leading to changes in UV absorption of 1 to 28^{72, 106, 111}. The intensity of the difference spectra is dependant on the extent of binding. Therefore, the association constants and thermodynamics of sugar binding to PNA can be determined by UV difference spectroscopy^{72, 106, 111}.

Matsumoto and coworkers¹⁰⁶ used this technique on various lectins and their specific saccharides and found an equilibrium constant of $2 \times 10^3 \text{ M}^{-1}$ for lactose-PNA interaction at 21°C. Using the same technique, Neurohr *et al.*¹⁰⁸ found binding constants at 25°C of $1.8 \times 10^3 \text{ M}^{-1}$, $1.3 \times 10^3 \text{ M}^{-1}$, and $1.0 \times 10^3 \text{ M}^{-1}$ for methyl- α -D-galactopyranoside, methyl- β -D-lactoside and methyl- β -D-galactopyranoside, respectively. The equilibrium constants, determined later by nuclear magnetic resonance, were in good agreement with those from UV-difference spectroscopy^{107, 108}.

Observed line-broadening effects on the NMR of ^{13}C -enriched sugar residues occurred upon specific binding of the glycosides to the peanut lectin^{107, 108}. The thermodynamic behavior and kinetic data, for binding to PNA, was determined for methyl-, α - and β -galactopyranoside¹⁰⁷ and methyl- β -D-lactopyranoside¹⁰⁸, using NMR. Both UV and NMR studies indicated a binding mechanism more complex than a simple bimolecular process^{72, 107, 108}. There is probably a ligand-induced conformational change in the protein, upon sugar binding to simple saccharides^{72, 108}. Neurohr and coworkers^{107, 108} proposed a two step binding mechanism, in which the sugar ligand binds to the lectin by forming an initial complex at a near diffusional rate. This is followed by a conformational change, to produce the final complex.

Similar UV-difference spectra, association activation entropies and association rates have been found for the galactopyranosides and lactopyranoside^{72, 107, 108}. This suggests similar binding mechanisms for mono- and disaccharides.

Thermodynamic data from NMR studies reveal a larger dissociation activation enthalpy and total enthalpy change for binding of the disaccharide, as compared to the monosaccharide, to the peanut lectin^{9, 42, 43}. This, once again, suggests an extended carbohydrate binding site on peanut lectin, as

initially indicated by hapten-inhibition studies^{59, 60, 108}.

"It is probable that both pyranosyl moieties of methyl- β -lactoside are involved in interactions with the residues of the PNA-binding site, including hydrogen bonding, as well as, favorable nonpolar contacts". (Neurohr, 1982¹⁰⁸)

The extended binding site of PNA is probably preformed, to some extent, for binding more complex saccharides and glycoproteins. Circular dichroism analysis of PNA indicated that glycopeptides differed from simple sugars in the changes that they induced in the lectin's CD spectra¹¹². Asialofetuin glycopeptides (T antigen-expressing) induced much smaller changes, upon binding to peanut lectin, than those induced by simple sugars (galactose and lactose)¹¹². This may indicate a binding site prefitted for the complex oligosaccharides which allows for an easier lectin-ligand interaction. More intensive conformational alterations are required to bind the simple sugars, leading to less facile binding and a lower binding constant, K_a ¹¹². Although no thermodynamic studies have been done with the T determinant disaccharide/peanut lectin system to date, Neurohr et al.¹⁰⁸ extrapolated the binding constants of galactose and lactose, with the relative inhibition data of Lotan et al.⁵⁹ to estimate a binding constant to T antigen of $1 \times 10^7 \text{ M}^{-1}$ at 25°C. This value agrees well with association constants found for other lectin-glycopeptide interactions¹¹³.

8. Peanut Lectin as a Cell Marker

a) Lymphocyte subpopulations

The lymphoid system is comprised of numerous cell subpopulations (or subsets) such as T and B, mature and immature cells, with different functions in immunological processes. In spite of their functional heterogeneity, the various cells, are as a rule, morphologically similar and are often found together in the same lymphoid tissue⁹⁹. In the past decade, distinct surface antigens have been defined which distinguish lymphocyte populations. Lectins, particularly the peanut lectin, have proved to be valuable for the detection of and identification of lymphocyte surface markers and the separation of lymphocyte subpopulations¹¹⁴.

The T-antigenic determinant is carried on the surface of all lymphocytes but usually in the sialic acid-masked form¹¹⁵. Therefore, peanut lectin is unable to interact with most lymphocytes, unless they have been previously treated with neuraminidase⁸⁶. It was found, in 1976, that murine thymus T lymphocytes could be separated into two populations by peanut lectin without the need of neuraminidase treatment⁸⁶. The PNA-reactive cells are the cortical thymocytes, which comprise the majority of the thymus cells (80-90%)^{86, 115-121}. These cells are sensitive to radiation and hydrocortisone elimination, and are

immunologically immature¹¹⁹.

The peanut lectin also binds to small numbers (<20%) of cells in bone marrow, spleen, peripheral blood and lymph nodes^{119, 121}. The distribution of the peanut lectin in various organs, as mostly examined by fluorescent PNA, is summarized in Table 5 (mouse) and Table 6 (man).

The PNA-binding cells from mouse bone marrow are enriched in spleen-colony forming units¹¹⁹. A study of PNA binding during ontogenesis, revealed that cells which bind PNA appear very early during the development of liver, thymus and spleen¹¹⁵. These cells are possibly prothymocytes or lymphoid stem cells¹²². A much larger percentage (15-25%) of PNA reactive cells were found in human umbilical cord lymphocytes than those found in adult peripheral blood and the cells which bound PNA were found to be immunologically immature^{119, 121}.

These findings have all led to the suggestion that PNA binding is a marker for immaturity among cells of the T-cell and hemopoietic stem-cell series¹¹⁵. Since germinal centres in mice (Peyer's patches) and man (tonsils) contain a higher percentage of PNA positive cells, the lectin may also be a marker for immature B cells¹¹⁸. Monocytes have also been found to bind PNA and are largely responsible for any peanut lectin binding which occurs in the peripheral blood lymphocytes¹¹⁹.

TABLE 5
Distribution of Mouse Lymphoid Cells
Which Bind PNA in vitro

<u>Tissue</u>	<u>Reference</u>	<u>Percentage of tissue cells which bind PNA</u>				
		<u>86</u>	<u>115</u>	<u>116</u>	<u>117</u>	<u>118</u>
Thymus		90	85	82	86	-
Peripheral lymph nodes		-	16	3.5	-	-
Spleen		15	6	5.5	13	4
Bone marrow		20	-	4.0	20	-
Peyer's patches		-	36	24	-	29
Peripheral lymphocytes		-	-	-	25	-
Foetal liver		-	-	-	19	-

TABLE 6
Distribution of Human Lymphoid Cells
which bind PNA in vitro

<u>Tissue</u>	<u>Reference</u>	<u>Percentage of tissue cells which bind PNA</u>		
		<u>119</u>	<u>120</u>	<u>121</u>
Thymus		70	52	50
Spleen		-	5	-
Tonsils		-	14	13
Peripheral blood		1	1	1
Cord blood		18	-	24

Rose et al.¹¹⁸ tested the binding of peanut lectin to cryostat sections of the lymphoid tissue from man, mouse, rat, hamster, guinea pig, rabbit, sheep and chicken. The results demonstrated that PNA binding to lymphoid tissue is very species dependant. Mammals which bound PNA strongly (man, mouse and sheep) showed a similar pattern of binding, namely, PNA bound predominantly to the thymic cortex and germinal centres¹¹⁸. These two tissues share other biological features such as: mitotically active cells, common cell death and the cells are outside the pool of recirculating lymphocytes¹²². The lymphoid tissue of hamster, guinea pig and rabbit did not bind PNA and the rat showed weak binding of peanut lectin in the thymic cortex and germinal centres. Neuraminidase-treatment of tissues not initially binding PNA, resulted in exposure of strongly PNA-reactive cells¹¹⁸.

PNA has also been employed to distinguish preleukemic cells from end stage leukemia cells in mice¹²³. Preleukemic bone marrow and spleen cells were PNA reactive whereas end-stage leukemia cells did not bind PNA¹²³. This agrees with other evidence of a similarity between the surface markers of preleukemic cells and prothymocytes¹²².

In humans, pathologic cells of different kinds of leukemia varied in their PNA-binding properties¹¹⁹. Blast cells from most patients with acute lymphoblastic

leukemia, stem cell leukemia, and myeloid leukemia, were PNA-positive, along with several cases of Burkitt tumours¹¹⁹. In contrast, the lymphocytes of the majority of chronic lymphatic leukemia patients did not bind PNA¹¹⁹. The presence of PNA positive cells in the peripheral blood of 13 out of 25 patients with childhood acute lymphoblastic leukemia was also reported by Levin *et al.*¹²⁴. It was further suggested that a high level of PNA positive lymphocytes may serve as an indication for poorer prognosis among these patients¹²⁴.

The peanut lectin has proven to be very useful for the fractionation of both normal and leukemic cells. The advent of separation techniques, applying immobilized lectins, will greatly facilitate research concerning the functions and properties of various lymphoid cell populations³⁷.

b) Embryonal Carcinoma

Embryonal carcinoma (EC) cells, the stem cells of teratocarcinoma, have been useful for studying certain aspects of the early stages in mammalian embryogenesis¹²⁵. Immunological studies on mouse EC cells have revealed several cell surface antigens common to this tumour type and to cells of the early embryos¹²⁵. Many of these surface antigens undergo significant changes during in vitro differentiation¹²⁶. Often the location, distribution and relative

mobility of sugar residues (as revealed by lectin binding) are different at different developmental stages⁴⁴.

The peanut lectin was found to bind to mouse embryonal carcinoma cells and to detect subpopulations among multipotent embryonal carcinoma cells¹²⁷.

Following in vitro differentiation, these cells no longer bind PNA¹²⁷ and the glycopeptides, responsible for PNA binding, disappear from the EC cells¹²⁶.

Isolation and biochemical analysis of the PNA receptor glycopeptides revealed they contain galactose and N-acetyl-galactosamine residues^{126, 128}. The progressive disappearance of the PNA receptors and other surface antigens from the cell membrane of differentiating EC cells suggests that cell surface carbohydrates are altered during the differentiation process^{126, 127}.

However, when a neuroblastoma line was tested, it failed to show any significant changes, in the number of PNA binding sites, upon differentiation¹²⁹.

D. THE THOMSEN-FRIEDENREICH ANTIGEN AND ANTIBODIES

1. Presence of T Antigen

Over 50 years ago, Oluf Thomsen¹³⁰ described a phenomenon termed pan-agglutination, in which a certain "propagative agent" rendered red blood cells agglutinable by one's own and all human sera. Friedenreich¹³¹ discovered that the "agent" in question was a bacterial enzyme. The postulated enzyme was designated as "transforming", and the new receptor, accordingly, as "T receptor"¹³¹. Subsequently, the acquired property of the Thomsen-Friedenreich phenomenon became known as T agglutination.

The cause of the T phenomenon is an enzyme present in certain bacteria and viruses¹³¹, now known to be neuraminidase. The enzyme, also called the Receptor Destroying Enzyme (RDE) or sialidase, as well as acidic treatment, will release terminal sialic acid (N-acetyl-neuraminic acid) residues and unmask a concealed cryptantigen, T antigen¹³³. Enzymatic T activation of human and blood cells may occur transiently in vivo during hematogenous infections due to in vivo unmasking by neuraminidase¹³⁴.

The T antigen is present in the masked form on all human erythrocytes⁶⁶ so that unmasking by neuraminidase allows the red blood cells to become agglutinated by anti-T present in all human sera¹³⁵. The T specificity is apparently not demonstrable on healthy untreated mammalian tissues¹³⁶ except in trace amounts in a brain

ganglioside, GM₁, whose T activity amounts to less than 0.5% of ordinary T antigen¹³⁶.

2. Anti-T Antibodies

The naturally occurring antibody, reactive with the T receptor, belongs mainly (55%) to the IgM class of globulins, up to 30% is IgA and significant quantities of IgG may also exist^{135, 136}. The origin of humoral anti-T is believed to be predominantly due to continuous antigenic stimulation by an individual's intestinal flora¹³⁷. This speculation has been based on the finding of T specificity expressed among many Enterobacteriaceae¹³⁷. Changes of gastrointestinal flora, induced by oral antibiotics, have been found to alter anti-T sera levels¹³⁸. In addition, oral administration of E coli O₈₆, possessing T activity, has recently been found to induce de novo production of anti-T¹³⁹.

3. M-, N-, T-, Tn- Glycoproteins

Although T specific structures occur in the exposed form in many microbes, such antigenic determinants are not available in the reactive form on most human healthy or benign tissues¹³⁷. They are uniformly shielded by O-glycosidically-linked carbohydrates such as sialic acid¹³³. The T antigen is the immediate precursor of the major antigens of the second human blood group system, MN¹⁴⁰. The determinant group of the T antigen was shown to be the

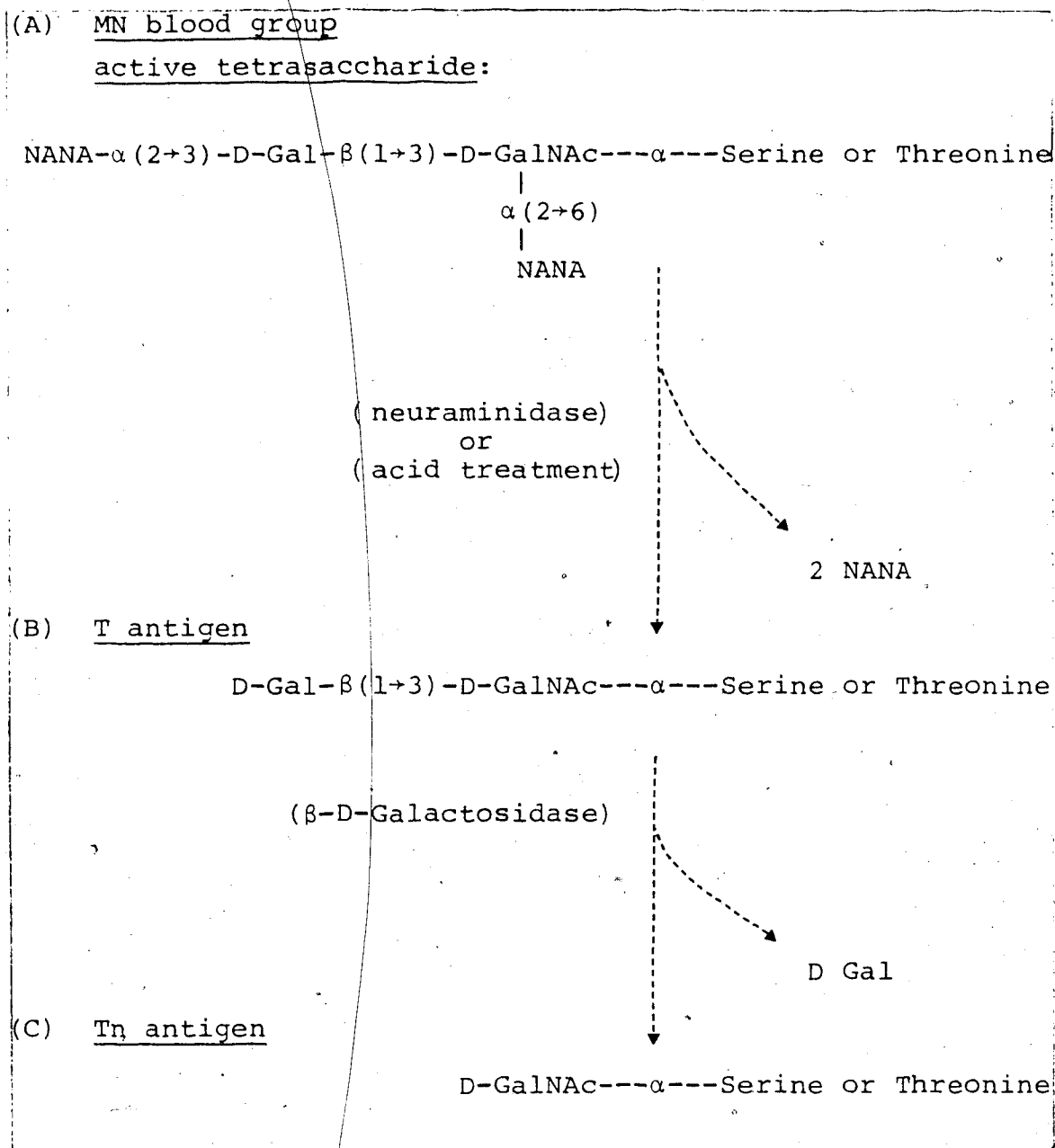


Figure 2: Structure of MN blood group active tetrasaccharide and precursor T- and Tn- receptors.

Key: NANA = N-acetylneuraminic acid; D-Gal = D-galactose;
 D-GalNAC = N-acetyl-D-galactosamine; α/β = anomeric configuration.

- After ref. 43 and 155.

disaccharide, β -D-galactosido(1 3)-N-acetyl- -D-galactosamine (BDGal(1+3) α GalNAc)(4) (see Fig. 2).

T antigens prepared from N, M or NM antigens have the same overall composition and react equally in hemagglutination inhibition assays with anti-T from humans and animals or with peanut (Arachis hypogoea) lectin^{140, 143}. Both peanut lectin and anti-T have been used in hemagglutination and precipitation methods of screening for T antigen⁶¹.

Further enzymatic degradation of the immunodeterminant disaccharide, by α -D-galactosidase, inactivates the T antigen, and reveals another precursor antigen, designated as the Tn antigen. Tn specific structures terminate in N-acetyl-D-galactosamine (GalNAc) which is α -glycosidically linked to the serine or threonine residues of the glycoprotein^{137, 142}. The relationship between these blood group antigens is shown in Fig. 2.

4. In Vivo Glycoprotein Synthesis

Current views on the transmembrane assembly of membrane and secretory glycoproteins have recently been reviewed¹⁴⁴. The peptide core of both secretory and membrane-associated glycoproteins is synthesized in the rough endoplasmic reticulum of the cell.

Synthesis of the O-linked oligosaccharide chains occurs as a sequential addition of single glycosyl units to the protein core. These additions are catalyzed at great speed by specific saccharide glycosyl transferases, located in the smooth endoplasmic reticulum and Golgi

apparatus. Sequential capping, with N-acetyl galactosamine, galactose and then sialic acid, all occurs in the Golgi apparatus before the Golgi-derived vesicles transport the finished glycoproteins to the plasma membrane¹⁴⁴.

Only the final sialic acid-masked product is believed to come in contact with the host's immune system¹⁴⁵. Biosynthetic intermediates, such as the T and Tn antigens, upon exposure would be recognized as "foreign" and could lead to immunological responses¹³⁷. In fact, neuraminidase has been used clinically, in tumour immunotherapy, to enhance a patient's own specific immunological reactivity against the exposed uncovered tumour antigens¹⁴⁶.

5. Composition and Structure of the T Antigen

Compositional and structural aspects of the precursor T antigen, isolated from erythrocyte MN antigens, are reasonably well known. Klenk and Uhlenbruck¹⁴⁷ isolated the membrane glycoprotein in 1958 from bovine erythrocytes. Springer et al.¹⁴⁸ isolated the T antigen from the O, NN antigen of human red blood cells. They found the T antigen to be an aggregate of repeating, apparently identical subunits with a molecular weight of 550,000 daltons. It was about 40 per cent carbohydrate, in which Gal and GalNAc predominated. The protein portion was rich in threonine, serine and glutamic acid and contained few aromatic and S-containing amino acids¹⁴⁸. The intact O, NN antigen glycoprotein contains about 17% neuraminic acid from the

80% total carbohydrate and 20% protein composition¹⁴⁴. The immunodeterminants of the glycoproteins and glycopeptides resist autoclaving at pH 7.0 and formaldehyde (pH7) fixing. This indicates the lack of protein involvement in the immunodeterminant activity.

The most active inhibitors of human anti-T and peanut lectin, found so far, are β -D-Gal(1+3) α -D-GalNAc^{62, 137, 143} and some desialized oligosaccharide fractions isolated from blood group M- and N- specific glycoproteins¹⁴⁸. The conclusion that this structure was the determinant group of the T antigen was further supported by the fact that Thomas and Winzler¹⁴⁹ also found this disaccharide to be the basic structure of the major alkali-labile sugar chains of the human MN blood group active glycoproteins.

T antigenic determinants also occur as part of carbohydrate structures other than MN specific substances. T as well as Tn, N and M structures have been found linked to lipid instead of protein in epithelial tissues such as salivary and breast glands and kidneys¹⁵⁰.

6. Cells and Tissues Expressing the T Antigen

The immunodeterminant T disaccharide structure can be carried by glycoproteins, lipopolysaccharides and glycolipids¹⁵¹. It is present in the masked form in a wide range of membrane-integrated and soluble glycoproteins, not only from vertebrates, but also invertebrates. It has been found, serologically and chemically, as a cryptantigen on

the membrane of erythrocytes⁶⁶, lymphocytes⁶⁶, thrombocytes²², kidney glycolipid¹⁵¹, and serum glycoproteins⁶⁶. This antigen has also been demonstrated in an exposed form, ie. not covered by sialic acid, for instance in "antifreeze" glycoproteins¹⁵², milk fat globule membranes¹⁵³ and in tumour cells^{151, 154}. Further occurrences of the T antigen on glycoproteins and glycolipids are illustrated in Table 7 and are discussed in current reviews¹⁵⁵⁻¹⁵⁷.

7. Relationship Between Cancer and T Antigen Expression

a) T specificities in animal carcinomata

Although most normal mammalian tissues do not express the T antigen in an exposed form, chemical and/or serological studies have revealed its presence in a number of animal carcinomata.

i) TA3-Ha (mouse mammary adenocarcinoma)

TA3-Ha, a spontaneous mammary adenocarcinoma of strain A mice, was shown to contain high concentrations of surface sialoglycoproteins. Proteolytic digestion of viable cells by Codington *et al.*¹⁵⁴ released a high molecular weight glycoprotein fraction composed largely of N-acetyl-galactosamine, galactose, sialic acid, serine and threonine. This fraction was called epiglycanin (glycoprotein I) and was found to express the immunodeterminant structures of the T and NM antigens¹⁵⁴.

Table 7
Occurrence of T antigen on Glycoproteins and Glycolipids

Occurrence	established		reference(s)
	chemically	serologically*	
"antifreeze" glycoproteins (antartic fish)	+	+	152
GM ₁ brain ganglioside glycolipid	+	+	165
bacteria and their products (esp. Enterobacteriaceae)		+	137
serum cholinesterase	+	+	158
apolipoprotein C-III	+		161
human chorionic gonadotropin (β -subunit)	+		159
IGA ₁ myeloma globulin	+		160
submaxillary gland mucin (dog and pig)	+		162,163
ovarian cyst mucopolysaccharides	+		164
gastrointestinal tract surface epithelium and adherent mucus (rats and mice)		**(+)	166,167
human colloid breast carcinoma mucin	+		168
human milk fat globule membrane	+	+	153
TAJ-Ha tumour cells (mouse)	+	+	154
Line 10 hepatocarcinoma (guinea pig)		+	150
13672 rat mammary ascites adenocarcinoma		**(+)	169,170
human adenocarcinomas and cultures (e.g. (e.g. breast, lung, GIT)		+	66,136,137
acute lymphoblastic leukemia cells and lymphomas		+	171
immature T lymphocytes and hematopoietic stem cells		**(+)	115

* T active disaccharide detected by anti-T reagents (antisera or Lectins).

** (+) detected by peanut lectin, which has an affinity, although much lower for residues other than T antigen, including simple galactose.

The TA3-Ha epiglycanin is carcinoma-associated and apparently does not occur in normal strain A mice^{154, 173}. Intact TA3 cells and the ascites fluid from the TA3 tumour-bearing mice absorbed the anti-N lectin, Vicia graminea, and human and animal anti-T¹³⁷. Purified human anti-T, in the presence of guinea pig complement, killed TA3 cells and this effect could be abolished by either T antigen inhibition or β -D-galactosidase pre-treatment¹⁷².

ii) ASPG-1 (rat) mammary adenocarcinoma

The major sialoglycoprotein found on cell surfaces of ascites k3762 rat mammary adenocarcinoma is a mucin-type molecule, called ASPG-1¹⁶⁵. It contains galactose, N-acetyl galactosamine and N-acetylglucosamine as the major sugars but has a composition differing from epiglycanin. Glycoprotein analysis, with ¹²⁵I-PNA, revealed that both the ascites cells and soluble ASGP-1, shed in the ascites fluid of tumour-bearing rats, carried the T-antigen (PNA receptor)¹⁷⁰. The solid form of the tumour, from which the ascites form was derived, was found to be lacking ASPG-1 PNA receptors¹⁷⁰.

iii) Guinea Pig Hepatocarcinoma

T specific structures have also been found in the lipid extracts of line 10 hepatocarcinoma of strain 2 guinea pigs¹⁵⁰. It was found that injections of gram negative bacterial extracts (which possess T-specific

structures) were able to cause regression of line 10 hepatocarcinoma in guinea pigs¹⁷⁴. This prompted investigation for the possession of MN and precursor T and Tn specificities on Line 10 tumour cells. Human anti-T sera showed that the immunoreactive T and Tn specificities were exposed on the surface of these cells¹⁵⁰. Normal glandular tissues of strain 2 guinea pigs were found to contain M and N but no T and Tn activities. The specificities were determined to be associated with a glycolipid, rather than a glycoprotein fraction since they were resistant to boiling and were extracted primarily into the organic phase of an n-butanol-water extraction¹⁵⁰. The T activity was found to be 5-10% of that of human red blood cell-derived T antigen¹⁵⁰. The presence of T- and Tn-specific structures in line 10 adenocarcinoma but not in healthy guinea pig glands agrees well with the situation in man¹³⁷.

b) T specificities in human carcinomata

The initial finding of T specificities in murine mammary adenocarcinoma prompted Springer et al.⁶⁵ to examine normal and cancerous human mammary tissue for the presence of MN antigens and their precursors. They showed with human antisera that, in contrast to healthy tissues, T and Tn-antigenic specificities do occur in the reactive unmasked form in human breast adenocarcinoma¹⁷⁵. The T and Tn antigens were therefore

postulated to be carcinoma-associated antigens¹⁷⁵.

Since Springer's initial discovery, a large array of malignant, benign and healthy tissues and cell cultures have been examined, by absorption assays for the presence or absence of T and Tn-specific antigens¹⁷⁶. The absorption of the anti-T by the tissues is often measured by the decrease in anti-T titre. The titre expresses the intensity of a serological reaction, such as hemagglutination, and is often quoted as the reciprocal of the last serum dilution giving the antibody-mediated reaction⁹⁹. A decreased titre indicates less agglutinating units per volume sera.

Anti-T sera (initial hemagglutination titre of approximately 14 to N'RBC) were absorbed with isolated glandular and cellular membranes. Absorption of anti-T, as determined by a decrease in agglutination score, resulted in the designation of the tissue as T-positive if a 34-100% (average 58%) decrease in score occurred⁶⁶. The results of some carcinoma and control tissues is illustrated in Table 8.

The T specificity was present in 47 of 52 (90%) breast adenocarcinomata samples. Two of 21 benign tissues were designated as T-positive but were considered to be histologically premalignant. Carcinomata of the respiratory and gastrointestinal tracts also had T specificity which was not confined

Table 8

Absorption of Human Anti-T
by Human Normal, Benign and Malignant Tissues**

Tissue	T-positive*	T-negative*
<u>Breast Carcinoma (ductal)</u>		
Primary	34	5
Metastases (late, distant)	9	0
Tissue culture	4	0
<u>Breast Carcinoma (lobular)</u>		
Primary	9	0
<u>Benign or healthy breast tissue</u>	2	19
<u>Non-breast Carcinoma</u>		
Respiratory tract	4	1
Gastrointestinal tract	5	0
cervix uteri squamous cell	1	0
retromolar trigone squamous cell	1	0
<u>Melanoma</u>	0	4
<u>Glioglastoma</u>	0	1
<u>Benign Non-breast tumours</u>		
Parotid mixed tumour	0	3
Lymphoma	0	1
Neuroma	0	1
Tendon sheath, giant tumour	0	1
<u>Healthy Tissues</u>		
Fallopian tube	0	1
uterus; cervix uteri	0	2
lung	0	2
aorta; blood vessels	0	2
testis	0	1
stomach, one ulcer (autolytic changes?)	2	0
<u>Red Blood Cells</u>	0	6
<u>Red Blood Cells, neuraminidase treated</u>	1	0

* Determined by a decrease in agglutination score (=anti-T absorption).

Initial hemagglutination score = 14 to N'RBC.

T-positive = 34-100% decrease in hemagglutination score.

T-negative = 0-34% decrease in hemagglutination score.

** From reference 1976.

to adenocarcinoma but was also found in squamous cell and undifferentiated carcinomata^{66, 176}. T-antigenic specificity was not found in the four melanomata, one glioblastoma and seven benign non-breast tumours tested. All distant breast metastases examined, retained the T-antigen-adenocarcinoma association even 5 years after removal of the primary tumour^{66, 176}.

Some investigators failed to show significant changes in PNA or IgM anti-T titres after absorption with various malignant and nonmalignant tissues¹⁷⁷. This may be due to the insensitivity and subjectivity of doubling dilution titres³³ which were used for antibody assay in this study.

Nordquist et al.¹⁷⁹ and Anglin et al.¹⁷⁸ carried out studies on the BOT-2 human mammary carcinoma cell line. They discovered that when these cells were grown in tissue cultures, they normally released glycoproteins of M-, N-, T and Tn specificities into the culture medium¹⁷⁸. Antigenically similar proteins were found in the serum of female nude mice bearing BOT-2 tumours, but not in control-mice¹⁷⁹. Nordquist et al.¹⁷⁹ suggested that rapid membrane turnover, especially with increasing tumour mass, results in the shedding of antigenic material and failure to form completely synthesized glycoproteins.

Springer et al.¹³⁷ suggest that the common occurrence of T antigen on various malignant tissues,

and not on benign tissues, is due to either incomplete synthesis or accelerated degradation during malignancy.

c) Immunohistological Localization of the T Antigen

Results of absorption studies have been supported by histological examination of fixed tissue sections with peanut lectin and/or anti-T. Fischer et al.¹⁸⁰ initiated interest in this direction, when they examined 8 histological slices of human mammary carcinoma with fluorescein-isothiocyanate (FITC)-labelled lectins. All 8 histological slices of mammary carcinoma were reactive to FITC-PNA, while surrounding normal tissue did not fluoresce¹⁸⁰. Preabsorption of the lectin with neuraminidase-treated red blood cells prevented fluorescence of the tissue.

Later investigations have demonstrated that peanut lectin shows some binding in vitro to normal and benign tissues, as well as malignant breast tissues^{67, 177, 181-184}. The designation of the T antigen as a tumour-associated antigen was therefore questioned¹⁸².

The pattern of distribution and degree of binding was different though in the tissue slices of malignant and non-malignant breast. In normal and benign tissue, free T antigen sites tended to be confined to apical regions of mammary glands, and to the secretions and luminal surfaces of epithelial cells^{177, 181, 182, 184}.

In vivo, these regions are usually immunologically secluded, due to poor contact with blood and lymph¹⁸⁴.

Malignant tissue demonstrated a more intense and diffuse PNA distribution than found in normal and benign tissue^{177, 182-184}. In addition, the pattern of peanut lectin distribution appeared to be related to the degree of morphological differentiation¹⁸². Studies on rat and human mammary tissue have revealed there may also be a correlation of PNA binding in mammary carcinoma and the degree of hormone dependence of the cancerous tissue¹⁸⁵.

Howard et al.^{184, 186, 187} reported results of immunoperoxidase studies on histological breast slices, utilizing both human antisera^{186, 187} and peanut lectin¹⁸⁴. He found that anti-T was bound to a less significant extent than PNA in the luminal regions of malignant and nonmalignant breast glands^{184, 187}. The luminal membrane localization, though, is essentially "outside" the body and is in an immunologically-privileged site. On the other hand, PNA-reactive sites found in the cytoplasm of malignant tissue would be more accessible for in vivo binding following the cell death which usually accompanies neoplastic growth¹⁸⁴.

Results of immunohistological binding studies with peanut lectin must be viewed with caution when used to predict the in vivo localization of PNA. The methodology used in immunohistological binding studies should be scrutinized for steps which may lead to artifactual binding (false positives) or inhibit true

binding (false negatives). Some factors and precautions to be considered are:

I. Tissue or Cell Sample Treatment

- a) trypsin treatment (to recover monolayer cell cultures) cleaves off glycoproteins⁶⁸.
- b) formalin treatment (tissue fixation) formalin is usually acidic and masking sialic residues may be cleaved off by acid hydrolysis⁶⁸.
- c) tissue sectioning - reveals intracellular components where incompletely synthesized and unmasked glycoproteins in the Golgi apparatus are present¹⁴⁴.
- d) tissue sectioning - reveals immunologically privileged sites to which blood and lymph may have poor access.

II. Specific Localizing Reagent

- a) lack of affinity purification - peanut extracts contain anti-Gy. Gy is a blood group factor inherited as a simple Mendelian trait with a population frequency of about 23%¹⁸⁸.
- b) reagent specificity - peanut lectin binds to residues other than the T antigen, although with a much lower affinity⁵⁹ and a binding spectrum very similar to anti-T¹⁸⁹.
- c) specific binding controls - inhibition by galactose.

III. Non specific binding of labelling reagent

- a) controls - necessary to ensure localization of the labelled reagent is not due to nonspecific binding of the label (eg. FITC, ferritin, immunoperoxidase).

Many of these precautions should be considered when examining the numerous studies utilizing PNA to examine histological breast sections. The relevance of each of the preceding problems (as denoted by I a) through III a)) for the various breast histological studies utilizing PNA, is shown in Table 9. The methodology of sample preparation, PNA detection and the sites of PNA reactivity are also illustrated.

d) Immune Status Towards the T Antigen
Carcinoma Patients

i) Humoral immunity

If serologically active T-specific structures are available in human adenocarcinoma, it may be expected to change the anti-T titre scores of human sera. The titre scores of 3 pooled control sera (36-1000 different sera) averaged 22-24 and was found to be depressed (to 12 or less) in a highly significant number of breast carcinoma patients¹⁹⁹ (Table 10). Similarly, patients with gastrointestinal (G.I.) carcinoma had depressed anti-T levels more frequently than patients with benign G.I. disease and noncarcinomatous control persons¹⁹⁰ (Table 10). Similar results were obtained by other investigators¹⁹¹.

Table 9 Immunohistochemical Studies of T Antigen in Mammary Tissue

Study	Tissues/Cells Preparation	Method Localization	T reactive sites (not neuaminidase treated)	Possible Problems* inherent in Methodology
Fischer 1977(180)	cyostat histological slices	FITC-PNA	-mammary carcinoma cells (and not surrounding normal tissue)	Ic), Id), IIIa)?, IIb), IIIa)
Klein 1978(181)	1) cell suspensions 2) formalin-fixed paraffin embedded 3) frozen tissue sections	1) PNA-RBC rosettes 2) H ³ -PNA 3) Fluorescein-PNA	-secretions and surface layer epithelial cells of lobuli and ducts in normal mammary tissue	Ib), Ic), Id), IIIa)?, IIb), IIIa)
Newman 1979(177)	1) fixed paraffin embedded (frozen tissue & sections)	1) H ³ -PNA 2) FITC-PNA	-acinar-epithelial cell surfaces and secretions of normal mammary tissue -diffuse and extensive distribution in carcinoma	Ib)?, Ic), Id) IIIa)?, IIb) IIIa)
Klein 1979(182)	1) formalin-fixed paraffin embedded (& frozen tissue sections)	H ³ -PNA FITC-PNA	-as for Newman 1979 -more differentiated carcinoma had increased and more diffuse binding over undifferentiated	Ib), Ic), Id) IIIa)?, IIb) IIIa)
Newman 1979(67)	1) formalin-fixed paraffin embedded (& frozen tissue sections)	H ³ -PNA FITC-PNA	-as for Klein 1979 -mature and lactating normal rat mammary lumina - (only cryptic in monolayer rat mammary stem line - differentiated)	Ia), Ib), Ic), Id) IIIa)?, IIb) IIIa)
Scheiffarth 1979(183)	frozen tissue sections	FITC-PNA	-tumour cell membrane -normal breast tissue also had a less intense membrane fluorescence	Ic), Id) IIIa)?, IIb) IIIa)
Howard 1979(186)	formalin-fixed paraffin embedded	human antisera (not affinity purified) -immunoperoxidase	-significant binding malignant (but not benign or healthy)	Ib), Ic) IIIa), IIc)
Howard 1980(187)	formalin-fixed paraffin embedded	-as for Howard 1979 -also affinity purified human antisera	-as for Howard 1979 -qualitatively same binding for purified antisera	Ib), Ic)
Howard 1981(184)	formalin-fixed paraffin embedded	PNA immunoperoxidase	-benign showed luminal membrane and secretion binding -diffuse cytoplasmic binding in malignant	Ia), Ic), Id) IIIa), IIb)
Klein 1981(185)	formalin-fixed paraffin embedded	FITC-PNA	-correlation hormone dependence and PNA binding in mammary carcinoma	Ib), Ic), Id) IIIa)?, IIb) IIIa)

* refer to text (pg. 72-73) for explanation.

**2 failure to specify in description of methodology.

Table 10
 Anti T Agglutination Titre Scores*
 in Carcinomatous and
 Non-carcinomatous Patients

<u>Diagnosis</u>	<u>Depressed anti-T Score \leq 12**</u>	
	<u>\bar{x}</u>	<u>No. of Persons</u>
<u>Carcinoma</u>		
Breast	32.3	61/189
GI	42.1	32/ 76
Lung	47.4	9/ 19
Total	35.9	102/284
<u>Benign Disease</u>		
Breast	8.2	22/270
GI	16.7	6/ 36
Lung	12.5	1/ 8
Total	9.2	29/314
<u>All Non-carcinomatous persons studied</u>	8.3	39/470

* (information taken from reference 190)

** (control titres 22-24)

Anti-T titres may be valuable for serial monitoring of breast carcinoma patients¹³⁷. When patients were re-evaluated 2 to 6 months following breast surgery, 21 of 32 (66%) of the mastectomy patients showed an increase of >25-90% in anti-T score (including patients with initially normal anti-T levels at time of mastectomy. In contrast, only one of 32 (3%) of the patients with benign disease had an increased anti-T level¹³⁷.

Although Springer and colleagues¹⁹⁰ could detect a significant change in anti-T levels in the presence of adenocarcinoma, they could not find a significant correlation with the extent of disease burden. Bray and coworkers¹⁹² utilized a more sensitive (complement-dependant cytotoxicity) assay to measure anti-T levels in patients with metastatic gastrointestinal cancer and did find a correlation of anti-T levels to extent of disease. Overall, 40% of the patients with metastatic gastrointestinal cancer had lower than normal levels of lytic anti-T in their serum. Low anti-T levels were found in 61% of patients with extensive disease, as compared to 27% and 9% in patients with moderate and minimal disease, respectively¹⁹². It is, therefore, possible that the low level of serum anti-T, in some patients with extensive disease, is due to absorption of the antibody by the tumour itself or by antigens shed into the circulation.

ii) Cellular Immunity

The majority of bacterial infections and immediate-type hypersensitivity reactions rely upon antibody production and humoral defenses. Cell-mediated immune responses are, at times, initiated by intracellular parasites (eg. mycobacteria, viruses and some protozoa) and are thought to play a role in immune reactions against malignant tumours. Cell-mediated immune responses involve the direct role of lymphocytes and associated cells but are intimately interdependent with the humoral immune system⁹⁹.

It has been found that cell-mediated reactivity towards the T antigen of breast carcinoma patients is demonstrable in vivo as well as in vitro¹³⁷.

A delayed-type hypersensitivity (DTH) reaction involves cellular immunity, and the prototype for it is the classic tuberculin reaction⁹⁹. Breast carcinoma patients have been shown to exhibit cellular immunity towards the T antigen^{66, 193} as summarized in Table 11. Following intradermal injection, in the arm contralateral to the breast lesion, these patients exhibited DTH reactions to human erythrocyte-derived T-antigen⁶⁶. The DTH response to T antigen was 78% sensitive (true positive) in all of the breast carcinoma patients tested. A sensitivity of 100% (no false positives) and 93% (7% false positives) was found for healthy persons and patients with benign breast disease,

Table 11

Delayed-Type Reaction upon i.d. Injection of T antigen and in vitro Cell-Mediated Immune Response to T antigen in Patients with Breast Carcinoma, Benign Breast Disease and in Healthy Controls

Disease Stage (Int'l Nomenclature)	<u>in vivo</u>		<u>in vitro</u>	
	Delayed-type-hypersensitivity Positive/Total tested		Leukocyte Migration Inhibition Positive/Total tested	
IV	17/17		9/17	
III	12/17*		11/25	
II	26/29*		18/35	
I	47/68*		17/47	
Total	102/131* (78%)		55/125 (44%)	
Benign	11**/147 (7%)		18/110 (16%)	
Healthy	0/77 (0%)		1/104 (1%)	

* The majority of negative tests occurred in lobular or tubular carcinoma (vs. ductal) patients.

** All but one had hyperplastic disease

- (compiled from information in references 66, 176, 190, 193)

respectively.

The sensitivity varied with cancer type, when the carcinoma patients were separated into those with the rarer and less ominous lobular and tubular carcinoma and those with ductal carcinoma. Fifty-two of 56 (93%) ductal breast carcinoma patients with Stage II-IV gave a positive response as compared to 13 of 28 (46%) and 0 of 2 patients with lobular and tubular carcinoma respectively⁶⁶.

The DTH reaction to T antigen was positive in all 25 lung adenocarcinoma patients tested and in 9/9 patients with adenocarcinoma of different origin (pancreas (3/3), esophagus (2/2), ovary (1/1), colon (2/2), and salivary gland (1/1) adenocarcinoma). All 7 patients with small cell (oat cell) carcinoma and 3/5 with malignant melanoma gave a positive response, whereas none of 17 patients with malignant brain tumours, leukemia or Hodgkins's disease, sarcoma or thyroid carcinoma reacted. The DTH reaction to T antigen appears to be a relatively accurate and specific test for T-antigen-bearing cancers⁶⁶.

One of the methods utilized to test cellular immunity in vitro involves measuring the inhibition of leukocyte migration (LMI) in the presence of specific antigens⁹⁹. Only lymphocytes specific to the particular antigen, upon contact with the antigen, will release chemostatic factors to inhibit migration of

macrophages and lymphocytes⁹⁹.

The results of this in vitro cellular immunity test are shown in Table 11. 38 of 77 (49%) patients with breast carcinoma, Stages II, III, or IV, showed positive in vitro reactions, as compared to 16% in benign disease patients, and 1% in healthy persons¹⁷⁶. Positive responses were not evident in the 5 malignant melanoma patients and the 1 glioma patient tested¹⁷⁶.

Among 7 patients with adenocarcinoma of lung, colon, rectum, esophagus, kidney and ovary, 3 had a positive reaction towards the T-antigen in the LMI assay¹⁷⁶.

In conclusion, the skin test with T antigen appeared to be more reliable than the in vitro test (which is carried out under very artificial conditions) for the detection of cell-mediated immunity to T-antigen in adenocarcinoma patients.

8. Galactose-Specific Tissue - "Lectins" and Metastases

It has been observed that neuraminidase-treatment of erythrocytes, lymphocytes, thrombocytes and serum glycoproteins leads to their rapid clearance from the circulation¹⁹⁴⁻¹⁹⁷. The desialylated galactose-expressing cells are entrapped by D-galactose specific lectin-like receptors on the surfaces of cells, such as hepatocytes¹⁹⁸, liver and spleen cells¹⁹⁴⁻¹⁹⁹ and peritoneal macrophages²⁰⁰.

The binding can be inhibited in vitro upon incubation of the "lectin" cells with galactose, oligosaccharides and glycoproteins (including desialylated glycophorin), all of which possess terminal galactose¹⁹⁹⁻²⁰⁰. The terminal β -galactose residues of asialofetuin and lactose can also block the in vivo sequestration of desialylated erythrocytes¹⁹⁹. The initial specific binding to macrophage lectins is believed to be necessary before the subsequent phagocytosis step¹⁹⁹.

It is possible that various body lectins could play a role in determining preferential localization of metastatic growth of carcinomas. The terminal sugar residues on the neoplastic cell surfaces could specifically interact with organ-associated lectins.

Metastases occur when a neoplasm reaches a certain critical size and it begins to shed cells into the bloodstream and lymphatic system²⁰¹. But considerably fewer than 0.01% ever give rise to any metastatic lesions²⁰¹. Usually the new growth begins in small capillaries where tumour cells are caught and begin to invade through the capillary endothelium. Certain tumours have a predisposition, usually due to anatomic location, to metastasize to certain organs²⁰¹.

The occurrence of specific lectins in vertebrates and in different organs of the human body led Uhlenbruck⁵⁰ to postulate a possible role in the mechanism of metastasis as shown in Fig. 3. Shed tumour cells carrying the

T antigen would carry terminal galactose residues and could preferentially localize in organs with β -galactose-binding lectins to metastasize in those organs. If further investigations are found to support this theory, it may be possible to block organ-lectin receptors with β -galactosidoglycoconjugates and thereby prevent organotropic localization of metastases.

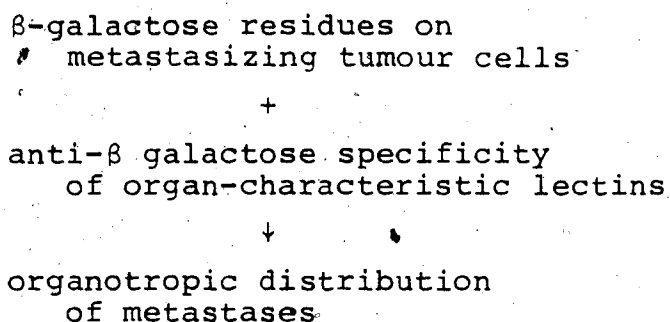


Fig. 3 Possible Role of Tissue Lectins in Organ Distribution of Metastases

E. RADIOIODINATION

Many parameters must be considered when choosing a radionuclide with which to label a radiopharmaceutical. The nuclear properties such as emission spectra, physical half-life and production factors must be considered, along with the effective half-life of the labelled radiopharmaceutical and the imaging device which is available²⁰². It is desirable to "maximize the detectable photon yield while minimizing the radiation dose to the patient"²⁰³. Ideally, the gamma ray energy should be between 100 and

250 keV, in order to allow maximum detection efficiency on the commercially available scintillation cameras. Within this energy range, the gamma rays will have sufficient tissue penetration but still be easily collimated and have good detection efficiency. In order to minimize the radiation dose to the patient, the radioisotope should lack non-penetrating emissions such as alpha and beta particles and low energy electromagnetic rays.²⁰² Ideally, the physical and biological half-lives of the radiopharmaceutical should contribute to an effective average half-life about as long as the duration of the diagnostic procedure. Other factors such as the cost, specific activity, radio-nuclidic impurities must also be considered²⁰².

1) Isotopes of Iodine

Radioiodines are commonly used for labelling proteins. The chemistry of iodine is well developed and the iodine forms a covalent chemical bond with the protein²⁰⁵. Upon degradation of the labelled protein, the radioiodine is not reincorporated into any tissue except the thyroid and this uptake can be blocked with potassium iodide²⁰⁵. There are 29 radioactive isotopes of iodine²⁰⁶ of which three, ¹³¹I, ¹²⁵I and ¹²³I, are in common clinical use²⁰⁷.

a) Iodine - 131

¹³¹I is readily available, inexpensive and emits 364 keV photons with an abundance of 80%²⁰⁶. This energy is slightly higher than ideal and additional

high energy photons, 637 and 722 keV, lead to septal penetration of collimators which may decrease the image sensitivity and resolution²⁰⁷. Unfortunately the radiation burden to the patient is relatively high with ¹³¹I, due to an 8 day half-life and the emission of beta particles during its decay. Although a specific activity of 4.6 GBq/μg is theoretically possible for ¹³¹I, a relatively low isotopic abundance in most preparations, results in much lower specific activities²⁰⁸. The most commonly used production method involves thermal neutron activation of tellurium (natural isotopic abundance). This produces a ¹³¹I preparation diluted with ¹²⁷I and contaminated by the long-lived ¹²⁹I isotope²⁰⁹. Uranium fission or the use of enriched tellurium targets may produce an ¹³¹I preparation which is less diluted by ¹²⁷I²⁰⁹.

b) Iodine-125

Iodine-125 is available in a high specific activity but is not used frequently for in vivo diagnostic procedures in humans. The low energy photons (28-35 keV) emitted by this isotope are readily attenuated by overlying tissue and contribute, along with a long physical half-life (60 days), to a high in vivo radiation dose²¹⁰. Iodine-125 has been used much more extensively in in vitro studies, due to a long shelf-life and the excellent detection efficiency for it in NaI well crystals²⁰⁸.

c) Iodine-123

Iodine-123 has recently attracted much attention in the field of nuclear medicine. ^{123}I has a short half-life of 13.3 hours and lacks primary particulate emissions²⁰⁶. The monoenergetic gamma emission at 159 keV is ideal for detection with modern gamma cameras and relatively large doses can be utilized in patients to ensure good resolution while minimizing the radiation dose absorbed by the patient²¹⁰. Long lived radionuclidic impurities, such as ^{124}I , which decrease image resolution and increase the radiation dose to the patient, make it necessary to limit the useful shelf-life to 24 hours²¹¹. Unfortunately, this nuclide is generally produced by means of an accelerator, which makes it expensive and limited in availability²¹⁰. In some instances, the physical half-life may be too short for imaging with radiopharmaceuticals which are not quickly localized in target tissues²⁰⁴.

2. Protein Radiolabelling

a) Direct Methods of Iodination

The most common methods of radioiodinating proteins are electrophilic substitution reactions in which the radioactive iodide is oxidized in the presence of a tyrosine containing protein²⁰⁸. The radioiodine is primarily incorporated into the tyrosine moiety of the protein, although

other amino acids such as: histidine, phenylalanine, tryptophan or sulfhydryl groups are sometimes iodinated as well^{203, 206}. The iodination of tyrosine moieties is illustrated in Fig. 4.

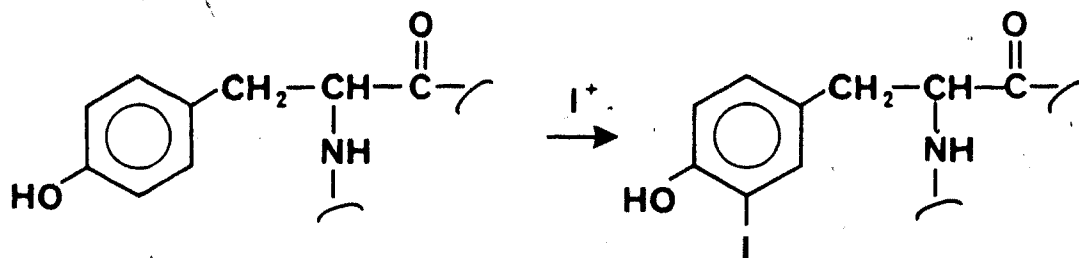


Fig. 4 Iodination of tyrosine moieties in proteins

Certain tyrosine residues may be more reactive than others in the iodination reaction, depending on the accessibility and microenvironment of the residues²⁰⁸. Several methods of radioiodination are available. Each method has certain advantages and may be more applicable for particular molecules; although limitations exist for each method. It is essential to establish the various parameters with each method in order that the optimum conditions can be attained for a particular radioiodination process. The following section contains a brief general description of several commonly employed radioiodination techniques.

i) Chloramine-T

Chloramine T, the sodium salt of N-chloro-p-toluenesulfonamide, was used as a radioiodinating

reagent by Greenwood and Hunter in 1963²¹². Excess chloramine-T in the presence of water, will form hypochlorous acid, HOCl, which then oxidizes iodide to cationic iodine, I^{+208} . Preferential substitution of the I^{+} occurs at the ortho position of the phenolic ring of tyrosine. The optimum pH for the chloramine-T reaction is around 7.5, so that the NaOH in the stock Na*I radioiodine should be buffered before use and the reaction should be carried out in the presence of an appropriate buffer, pH 7.5²⁰⁸. Sodium metabisulfite is added to stop the reaction by reducing iodine back to iodide and reducing the excess chloramine-T²¹³. Because the oxidizing and reducing steps may damage the protein, concentrations and durations of exposure should be kept to a minimum²¹⁴. The chloramine-T reaction is technically simple and the reactivity allows products of high-specific-activity to be rapidly prepared²⁰⁸.

ii) Iodine Monochloride

The method of iodination by iodine monochloride was initially developed by McFarlane²¹⁵ and later modified for labelling micro quantities of proteins²¹⁶. Iodine monochloride is mixed with radioiodine in order that the exchange reaction can occur to form *ICl. The *ICl then acts as an electrophile, upon the addition of the protein solution²¹⁷. The radioiodination reaction is terminated by the addition of excess

sodium metabisulfite, to reduce free radioactive iodine to iodide.

The ^{127}I carrier, added in the form of $^{127}\text{I}-\text{Cl}$, is substituted to a certain degree into the protein. Therefore, the maximum specific activity which can be attained with iodine monochloride is lower than with the Chloramine T method²⁰⁸. A recoil method of preparing no-carrier added radioiodinated ICl, using chlorine was proposed by Lambrecht et al.²¹⁸ but the complicated techniques involved have negated common usage of this technique²⁰⁴.

The ICl, commercially available, often contains I_2 which will decrease the labelling yield²¹⁹. Therefore the ICl must often be synthesized by the investigator²¹⁹. The iodine monochloride method has the advantages of excellent control over the degree of iodine substitution resulting in generally good retention of biological activity in the radiolabelled protein.

iii) Enzymatic iodination

Lactoperoxidase is an oxidizing enzyme first used by Marchalonis²²⁰ for the enzymatic oxidation of iodide to iodine in the presence of hydrogen peroxide. Later, modifications by Thorell and Johanssen²²¹ increased the specific activities attained by enzymatic iodination. This method minimizes protein damage and loss of biological activity, because the protein is not exposed

to strong oxidizing and reducing agents²⁰⁸. The reaction is initiated by the addition of a small amount of hydrogen peroxide to a mixture of lactoperoxidase, radioiodine and protein. Further aliquots of hydrogen peroxide are added at 10 minute intervals until the reaction is terminated by either dilution or enzyme quenching with cysteine²⁰⁹. The pH, reaction duration and conditions must all be optimized for each protein and the purity and enzymatic activity of the lactoperoxidase must be determined. Removal of the enzyme from the radiolabelled protein has been greatly simplified by coupling the enzyme to an insoluble bead to facilitate separation²²². The large size of the enzyme tends to favor radioiodination of peripheral tyrosine residues rather than central residues²¹⁹. The yields are not usually very high, even though no-carrier added iodide can be used.

iv) Electrolytic Iodination

One of the gentlest iodination methods is electrolysis at a constant low current²²³ and the method has been modified for iodinations at the micro level²²⁴. The constant current electrolysis oxidizes the iodide at the anode to provide the iodine electrophile²¹³. The reaction is mild and produces homogeneous iodination with virtually no protein damage. Various types of electrolytic cells have been developed and used at different currents and voltages. The

labelling yield increases with time, at a rate dependant upon the reactant concentrations and operating current. Although labelling efficiencies of 80-90% can be attained, this method is not greatly used. Reaction volumes usually need to be larger than those required for other reactions²⁰⁴ and the complex electrolytic iodination technique requires a sophisticated micro-electrolytic cell²⁰⁶.

b) Conjugation Labelling

In most of the direct iodination methods the oxidizing agents may damage the protein²⁰⁸. Bolton and Hunter²²⁵ developed a method in which an ester is initially labelled and purified from all oxidizing and reducing agents before it is coupled to the protein²⁰⁸. It can be used for proteins which lack tyrosine residues or whose biological activity is damaged by iodination of tyrosine residues²¹⁴. The phenolic group of 3-(4-hydroxyphenyl) proprionic acid N-hydroxy succinamide ester (Fig. 5) is iodinated by the Chloramine-T method and then separated from the oxidizing agents²⁰⁸. The iodinated ester is coupled, by amide bonds, to epsilon amino groups of lysine or terminal amino acids of a protein molecule. Additional details and data on reaction conditions have been presented by Bolton^{208, 225}.

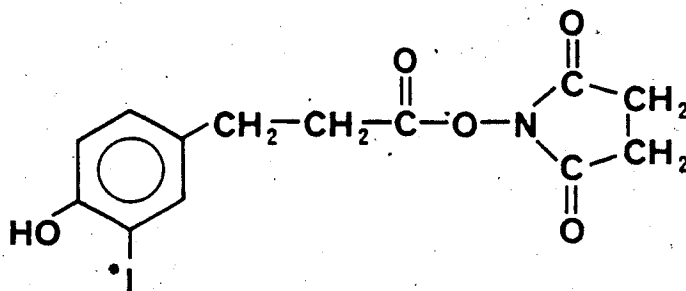


Fig. 5 Iodinated Bolton-Hunter reagent

The complexity, involved in the numerous steps of the conjugation labelling method, generally results in iodination yields and specific activities which are lower than direct iodination techniques. The protein is not exposed to radioiodine solutions, oxidizing or reducing agents during conjugation, so that the biological activity is usually maintained. Occasionally, the size of the conjugate or modification of amino residues may alter the biological activity²⁰⁸. Some other conjugates, besides the Bolton-Hunter reagent, which have been used are methyl p-hydroxybenzimidate hydrochloride and diazotized aniline²²⁷.

3. Protein Damage From Radioiodination Procedures

For a radiolabelled protein to be useful for tumour imaging purposes, it should be labelled to a high enough

specific activity to allow detection but still maintain the substrate specificity²⁰⁴. Chemical damage may occur during the iodination procedure because of the use of strong oxidizing and reducing agents. The radioiodine solution itself may oxidize sulfhydryl groups or cleave disulfide bonds in the protein²¹⁴.

The degree of iodine substitution is also important. When a large number of iodine atoms are incorporated into a protein, it may alter the biological activity of the protein. It is often most desirable to have an average incorporation of one iodine atom per protein molecule²⁰⁸. At low iodine incorporation levels, the probability of distribution of several atoms per protein molecule decreases.

Usually the damage that occurs during iodination is due to minor structural changes in the protein structure²¹⁴. The biologically active site of a protein makes up only a small proportion of the large macromolecular structure, so that alterations in protein structure do not always result in loss of biological activity. Therefore, it may not be an absolute requirement that all properties of the labelled proteins are identical to those of the unlabelled product. The slow blood clearance of proteins often makes the detection of target radioactivity difficult²⁰⁴. If the damage to the labelled protein results in very little loss of biological activity but leads to an accelerated blood clearance, earlier and more sensitive imaging may be possible²⁰⁴. This was demonstrated with heavily iodinated

fibrinogen, used to detect thrombi. When iodinated with 25 atoms/molecule, there was some loss in biological activity but there was also an accelerated rate of blood clearance. The end result was a larger thrombus:blood ratio of radioactivity and thus better imaging characteristics^{22B}.

Therefore all the factors, such as specific activity for localization, rate of blood clearance and retention of biological activity, must be examined when choosing an iodination method and the degree of iodine incorporation.

METHODOLOGY

A. PREPARATION OF PROTEIN SOLUTION

1. Anti-T IgG (rabbit)

The anti-T immunoglobulins used in these studies were kindly provided by ChemBiomed, University of Alberta. They used an artificial T antigen, produced by coupling the synthetic T disaccharide⁸⁹ to BSA, for the immunization of rabbits^{229, 230}. Following production of the high titre sera in the animals, anti-T IgG was affinity purified on an immunoabsorbent, also prepared from the synthetic T hapten⁸⁹.

The rabbit anti-T solution was provided at a concentration of 1.34 mg/ml in PBS (=phosphate buffered saline), (Gibco 0.01 M phosphate buffer in 0.15 M sodium chloride, pH 7.4).

2. Nonspecific Rabbit IgG:intact and F(ab')₂ Fragment

Chromatographically purified rabbit IgG (Cappell Lab., Cochranville, PA) was obtained in a lyophilized form with 0.1% azide preservative and was reconstituted with glass distilled water to a concentration of 25 mg/ml. The azide preservative was removed by 3 successive concentrations on an Amicon^R B-15 macrosolute concentrator (Amicon Corp., Mass.). Dilution with 0.4 M borate buffer, pH 7.4, was carried out between each concentration step. The protein and one wash solution were removed from the concentrator, added to 25 mg T immunoabsorbent (ChemBiomed, University of Alberta) and mixed by rotation gently for 2 hours at 4°C. The supernatant protein, and a 0.4 M borate buffer

wash of the T-sorb, were aseptically passed through a 0.22 μ M Millex-GS filter (Millipore Corp., Bedford, MA) into a sterile vial. The sterile IgG solution was aliquoted into sterile autoclaved ampoules and then sealed on a Cozzoli^R sealer before being stored at -15°C .

The rabbit F(ab')₂ IgG (Cappell Lab) was purified in a similar manner as the intact IgG although in 1 to 6 mg lots. The millipore filtered protein was aliquoted into sterile Falcon^R tubes (CanLab), stored at 4°C and used within 2-4 weeks.

3. Peanut Agglutinin

The peanut lectin was obtained from E.Y. Lab (San Mateo, Ca.) in salt- and sugar-free lyophilized form. The protein had been affinity purified (lactose-sepharose 4B column) to a pure single band on polyacrylamide disc gel electrophoresis.

The PNA was reconstituted with sterile PBS in a sterile Falcon tube to 1 mg/ml and passaged numerous times through a 26 gauge needle to break up the aggregates. The protein solution was then passed through a 0.22 μ millipore filter before being placed in a previously autoclaved (121°C x 20 minutes) 1 cm UV cell (Thermal syndicate Ltd., England). Optical density was measured at 280 nm in comparison to a PBS blank and the protein concentration was determined as described later. The solution was stored at 4°C , for no longer than two months.

B. QUANTITATIVE PROTEIN ANALYSIS

1. Rabbit IgG: Intact and F(ab')₂ Fragment

The protein concentration of the initially reconstituted antibody preparations was determined by a modification of the Folin-Phenol procedure^{231, 232}. A standard curve of absorbance versus antibody concentration was prepared using nonspecific IgG immunoglobulin (Cappell Lab.).

The copper reagent was prepared freshly and consisted of: 1 part 1% W/V copper sulphate (CuSO₄·5H₂O) 1 part 2% W/V aqueous sodium potassium tartrate and 20 parts 10% W/V sodium carbonate in 0.5 N sodium hydroxide. One ml of the copper reagent was added to one ml of the protein sample and following thorough mixing, it was incubated at room temperature for 10 minutes. Three ml of a 1:11 distilled water dilution of the Folin-Phenol reagent (Fischer Sci.Co., Fair Lawn, NJ) was then added, thoroughly mixed and incubated at 50°C for 10 minutes. The absorbance of the solutions was read at 640 nm on a Unicam SP1800 Spectrophotometer (Canlab) using a blank prepared in the same manner, but substituting PBS for the protein solution. A standard curve of increasing protein concentrations, from 1 µg/ml to 200 µg/ml, against optical density was then used to determine the sample concentration.

Separate standard curves were prepared for both the intact and F(ab')₂ fragment of IgG proteins using the same respective immunoglobulins (but non-specific IgG) as standards.

The protein concentration of the antibody solutions was also determined by ultraviolet absorption at 280 nm. The absorbance of the solutions, against a saline blank, was determined on a Unicam SP 1800 UV Spectrophotometer. The concentration of the immunoglobulin solutions was calculated by means of the absorbance coefficients of $E_{1\text{ cm}}^{1\%} = 14.6$ for intact rabbit IgG and $E_{1\text{ cm}}^{1\%} = 14.8$ for $F(ab')_2$ fragment IgG²³³.

2. Peanut Lectin

Optical density, at 280 nm, was also used for determination of peanut lectin concentrations, using the method described for antibody preparations. An absorbance coefficient of $E_{1\text{ cm}}^{1\%} = 9.6^{69}$ was used to convert absorbance readings to a concentration value.

C. RADIOLABELLING OF PROTEINS

1. Quality Control of Radioiodine Solutions

The radionuclidic purity of Na^{125}I solutions (Iodination grade, AECL) was confirmed using a 50 kBq aliquot in a $\text{NaI}(\text{Tl})$ well crystal and a multichannel analyzer (Canberra, Series 40 MCA) and comparing the gamma spectra obtained to that of a published standard²³⁴.

The radiochemical purity of the Na^{125}I was confirmed using instant thin layer chromatography. Twenty kBq was spotted on Gelman^R silica gel, instant thin layer 20 cm strips, air dried and developed in an 85% methanol in water

solvent system. A chromatogram scanner was used to analyze the strips for distribution of radioactivity.

2. Radioiodination Procedures

a) Protected Anti-T IgG Immunoglobulin

Anti-T rabbit IgG immunoglobulin was prepared by ChemBiomed, University of Alberta and graciously supplied for research purposes. Anti-T IgG (180 µg) was placed in a 3 ml Reacti^R vial (Pierce Chemical Co., Rockford, Ill.) with 60 mg T-sorb (ChemBiomed) and 1 ml 0.5 M phosphate buffer, pH 7.2. This mixture was gently rotated for 2 hours at 4°C. The T-sorb was washed (3x) with 1 ml aliquots of 0.5M phosphate (PO_4^-) buffer. Following removal of the final wash, the T-sorb-bound anti-T was labelled, in 1 ml of PO_4^- buffer, by a modified iodine monochloride method²⁶. Three to six µl of Na^{125}I (iodination grade, AECL) was added and the mixture was gently agitated for 30 seconds before the addition of 25 µl of a 1:50 dilution of stock iodine monochloride solution (3.4×10^{-3} M). The iodination of the T-sorb-bound antibody was stopped 3 minutes later. In initial labelling experiments, 25 µl of fixing solution (0.16 M $\text{Na}_2\text{S}_2\text{O}_3$ and 0.012 M KI) was added to stop the reaction. This step was eliminated in later labelling experiments. The supernatant was removed and 4 washes of the labelled antibody-T-sorb complex were carried out with PBS.

The anti-T was eluted off the T-sorb with 400 μ l of 2% NH_4OH and then returned to physiological pH (7.2-7.4) with saturated KH_2PO_4 . The labelled antibody was then either concentrated on an Amicon^R concentrator or purified on a Sephadex G-25M prepacked PD-10 column (Pharmacia, Sweden); both of which were pre-equilibrated with 1.5% bovine serum albumin (Sigma, St. Louis).

b) Nonspecific IgG Immunoglobulin

100 μ g of purified rabbit IgG immunoglobulin was labelled with ^{131}I (iodination grade, AECL) also by the iodine monochloride method. The protein was mixed with 3 μ l $\text{Na } ^{131}\text{I}$ and 1 ml 0.5M phosphate buffer for 30 seconds. The reaction was initiated upon the addition of 25 μ l of a 1:50 dilution of the stock ICl solution and terminated 3 minutes later with 25 μ l fixing solution. The labelled protein was separated by gel filtration on a Sephadex PD-10 column eluted with PBS.

c) Peanut Lectin and $\text{F}(\text{ab}')_2$ Nonspecific IgG

300 μ l of PNA (0.3 mg/ml in PBS) was buffered with 20 μ l of 0.5M phosphate buffer, pH 7.4, in a 3 ml Reacti-Vial. Following the addition of 3-6 μ l $\text{Na } ^{125}\text{I}$ (3.7 MBq/ μ l, iodination grade; AECL) the reaction was initiated by adding 30 μ l of Chloramine T (1 mg/ml in 0.05M phosphate buffer, pH 7.4). The reaction was terminated 30 seconds later, by the addition of 60 μ l sodium metabisulfite solution (1.2 mg/ml in 0.05M

phosphate buffer). In biodistribution studies utilizing $F(ab')_2$ simultaneously with PNA, 100 μ l (1 mg/ml) of each protein solution was placed in 0.3 ml reacti vials. The $F(ab')_2$ fragment and PNA were then labelled with I-131 and I-125, respectively, as outlined above. A Bio-Gel P-6DG column was used to remove free iodide.

3. Separation of Unreacted Free Iodide

a) Gel Filtration Media

i) Sephadex G-25 in PD-10 columns

Disposable prepacked PD-10^R columns of Sephadex G-25 medium were obtained from Pharmacia (Canada) Ltd. and used for purification of radiolabelled proteins in anti-T studies. These columns have a bed volume of about 9 ml and a void volume for proteins of about 2.5 ml.

ii) Bio-Gel P-6DG

Bio-Gel P-6DG^R (Bio-Rad Lab, Mississauga, Ont.) is a desalting polyacrylamide gel with an exclusion limit of about 6000 daltons. The gel was hydrated by incubating it overnight at room temperature in PBS. Twice as much PBS was used as the expected packed volume of 8 ml/gram Bio-Gel P-6DG. The column used in initial PNA experiments was a PD-10 column from which the Sephadex was removed. To improve resolution, a longer column, of about 11 ml packed bed volume, was prepared in a Pharmacia^R 9 mm x 300 mm column.

b) The Separation Procedures

To prevent non-specific adherence, 0.5 ml of 1% BSA in PBS was passed through the columns before application of radiolabelled proteins. The protein mixture (antibody or lectin) was layered on top of the bed surface and allowed to drain in, before PBS was added to wash the sample into the bed. The eluting PBS was added at a flow rate of about 0.4 - 0.5 ml per minute. The eluate was monitored for both protein and radioactivity. A flow-through ultraviolet photometer (LDC Duo Monitor) was set at 280 nm to detect protein absorbance in comparison to the PBS buffer in a paired UV cell. The thin polyethylene cannula, carrying the eluate, also passed across the face of a shielded 3" x 3" NaI(Tl) crystal detector attached to a single channel analyzer. A dual-pen Fisher Recordall 5000 recorder was used to record the simultaneous detection of radioactivity and protein optical density.

4. Determinations of Labelling Yield and Radiochemical Purity

a) Trichloroacetic Acid (TCA) Precipitation

Aliquots of reaction mixtures were taken before and after purification procedures and diluted to 1 ml with 1% BSA in PBS. Following the addition of 1 ml of 20% TCA, the denatured protein mixture was mixed on a vortex mixer and then centrifuged at 500 rpm for 10 minutes. After removal of the supernatant, the

precipitate was redispersed in 20% TCA before a second centrifugation (500 rpm x 10 minutes) was performed. The initial amount of radioactivity added had been assayed and following separations the activity in the supernatants and the precipitate was determined in an automatic gamma well-counter.

b) Instant Thin Layer Chromatography (ITLC)

Ten or twenty micro-litre aliquots from the reaction mixtures and purified protein were spotted on silica gel instant thin layer strips (Gelman, Ann Arbor, Michigan) and dried. The chromatograms were developed to approximately 15 cm in 85% methanol in water and then cut into 1 cm strips and assayed for radioactivity on a Beckman 8000 gamma spectrometer.

c) Gel Filtration Fractionation

A radioactivity elution profile was obtained during the gel filtration separation of unreacted radioiodide from radiolabelled protein. This provided information on the relative distribution of radioactivity in the reaction mixture. Analysis was carried out by either determining the area under the elution profile curves or by totalling the radioactivity of the fractions composing the salt or protein peaks. Nonspecific losses of radioactivity on the column were assumed to be loss of labelled protein, rather than free iodide. Earlier experiments had verified that free iodide is quantitatively

recovered from the column.

3. Radiochemical Stability of Iodinated Peanut Lectin

The rate of hydrolysis of I-125 from radiolabelled peanut lectin was determined. Fifteen μg of PNA protein was labelled by the iodine monochloride method utilized for the radioiodination of IgG (as described in section C2b) and 15 μg was labelled by the Chloramine-T method (section C2c). Free unreacted iodide was removed from both labelled products by gel filtration with a polyacrylamide Biogel^R P-6DG (Bio-Rad Lab, Richmond, Calif.)-packed 9 mm x 300 mm column which had been pre-equilibrated with 1.5% BSA. Protein fractions were collected in BSA precoated falcon tubes and then stored at 4°C.

Three 100 μl samples of each labelled protein were taken 0, 1, 2, 3, 4, 5, 6, 7, 10, 14 and 17 days following radioiodination and mixed with 900 μl 1.5% BSA in PBS. One ml of 20% trichloroacetic acid (TCA) was added to precipitate the protein, followed by centrifugation (500 rpm x 10 min.).

The initial mixture, and the separated precipitate and supernatant were analyzed for radioactivity on the Beckman 8000 gamma counter employing the I-125 dpm program. The percentage of radioactivity which was TCA precipitated was calculated by dividing the counts in the precipitate by the sum of activity in the precipitate and supernatant.

D. TUMOUR CELL CULTURING

1. Preparation of Tissue Culture Media

Five hundred ml of sterile fetal bovine serum (Gibco, Grand Island, N.Y.) was heat-inactivated, by a one hour water bath incubation at 57°C, and then aseptically aliquoted into 50 ml samples before storage at -20°C. RPMI 1640 medium (500 ml) with L-glutamine and 25 mM HEPES buffer (Gibco) was aseptically supplemented with a 50 ml aliquot of heat inactivated fetal calf serum and 5 ml of 200 mM L-glutamine (Gibco). The tissue culture media were stored at 4°C and used within 3 months.

2. Tumour Cell Culturing Procedures

All tumour culture procedures were carried out in a laminar flow hood (Baker Co., Sanford, Maine) which had been previously exposed to germicidal U.V. light and scrubbed with 70% isopropyl alcohol.

Suspension cultures of the mouse tumour cell lines RI lymphoma, EL4 lymphosarcoma and BW5 lymphoma were maintained in a carbon dioxide incubator (Hotpack^R, Waterloo, Ont.) at 37°C and a CO₂ concentration of 4 - 5%. To determine cell concentrations, aliquots were aseptically withdrawn from the cell suspensions and spotted onto a prepared hemocytometer (American Optical Co., Buffalo, N.Y.) and cells were counted at 150 X microscopic magnification. In viability determinations, trypan blue was added to the culture aliquot before microscopic examination and the

percentage of cells which excluded dye was calculated.

The mouse tumour cell lines were subcultured every 2 or 3 days. Tissue culture media was preheated to 37°C in a serological water bath (Fisher Scientific Co. Ltd., Fair Lawn, New Jersey). Cells ($1-5 \times 10^5$) were inoculated into 10 ml of preheated culture media contained in 25 cm² sterile tissue culture flasks (Corning^R, New York). The cells were recultured before a maximum cell concentration of 10^6 cells/ml was reached, in order to ensure log phase growth. Disposable serological borosilicate pipettes (CanLab), which were utilized for subculturing, were immersed in a bleach solution following use and culture flasks and tubes were autoclaved before disposal.

E. IN VITRO TESTS OF THE BIOLOGICAL ACTIVITY OF RADIOLABELLED PROTEINS

To prevent nonspecific adherence and protein losses in in vitro studies, tubes were pre-coated with bovine serum albumin (BSA). Three ml aliquots of 3% BSA in PBS solution were added to each 12 x 75 mm Falcon^R plastic tube, followed by incubation for one hour at 37°C and then for an additional 24 hours at 4°C. The BSA solution was removed and the tubes rinsed once, with 3 ml PBS. Tubes were capped and stored at 4°C until use. All binding studies were done in tubes precoated with BSA.

1. Studies with Neuraminidase-Treated Red Blood Cells
N'RBC

a) Neuraminidase treatment

The T antigen in human red blood cells was exposed by neuraminidase treatment. Red blood cells were collected from the defribinated blood of a group O, rhesus negative donor and used within 2 days of collection. A 2% (V/V) suspension of packed red blood cells in PBS was incubated for 45 minutes at 37°C with Vibrio cholerae neuraminidase (Gibco Ltd.), at a final concentration of 5 units/ml. Saline replaced neuraminidase for the preparation of control RBC. Following neuraminidase treatment, cells were washed by centrifugation (4x) with PBS and resuspended to a concentration of 2% (V/V).

b) Hemagglutination titre to N'RBC

Serial dilutions, of radiolabelled and unlabelled lectin, were carried out with PBS in BSA-precoated tubes. Equal volumes (100 µl) of diluted lectin and N'RBC were mixed and incubated at 4°C for 30 minutes. The mixtures were then centrifuged (500 rpm x 5 minutes), gently resuspended and examined visually for hemagglutination.

c) Binding studies to N'RBC

One ml of 2% N'RBC, or serial dilutions of a 2% suspension, and one ml of control RBC were placed in separate BSA-precoated tubes. The cells were incubated for 10 minutes at room temperature with 1%

BSA in PBS. Following centrifugation (500 rpm x 5 minutes) the supernatant was removed and 100 μ l of diluted radiolabelled antibody or peanut lectin was added to the cell pellets. The mixtures were resuspended, counted for total radioactivity and incubated on ice for 30 minutes. Three washes were performed with 1% BSA in PBS before the final pellet was recounted for bound radioactivity.

The specificity of binding was assured by inhibition of binding in the presence of galactose. For these controls, both the 1% BSA preincubation step and the added radiolabelled protein contained 0.1 M galactose. The 1% BSA used for washing (post-¹²⁵I-PNA incubation) contained 0.02 M galactose.

2. Studies with Synthetic Carbohydrate Immunoabsorbents

Lemieux, Ratcliffe and colleagues of ChemBiomed Ltd., University of Alberta, synthesized a number of synthetic carbohydrate immunoabsorbents^{89, 229, 230}. These "synsorbents" are composed of various mono-, di- or trisaccharides which are covalently linked by a 9 carbon branching chain to silylaminated, calcined, diatomaceous earth. ChemBiomed kindly supplied us with a selection of these immunoabsorbents for research purposes.

a) The T hapten (8 methoxy carbonyl octyl-2-acetamido-2-deoxy-3-O-(β -D-galactopyranosyl)- α -D-galactopyranoside) immunoabsorbent was synthesized

by Ratcliffe et al.⁸⁹. The solid support of the immuno-adsorbent was used as a control. 5 mg of both the T-sorb and synsorb blank were incubated with 0.5 ml 1.5% BSA for 10 minutes in BSA precoated tubes. Following removal of the BSA solution, 100 μ l of diluted radiolabelled antibody or peanut lectin was added and the total content of radioactivity was determined. The synsorb mixtures were incubated, with gentle rotation, at 4°C for one hour. Three washes were performed with 1.5% BSA in PBS before the immuno-adsorbents were counted for bound radioactivity (I-125 dpm program Beckman 8000).

b) Study of Carbohydrate-Specificity of PNA

To determine the specificity of binding, immuno-adsorbents prepared from other carbohydrates were incubated with I-125 PNA. Twenty mg of the various immuno-adsorbents were weighed out into BSA precoated Falcon tubes. The synsorbs were incubated with 0.5 ml 1.5% BSA for 10 minutes, before 100 μ l of ¹²⁵I-PNA (30 kBq/0.6 μ g PNA in 100 μ l PBS) was added.

The mixtures were incubated for 45 minutes at 4°C with continuous gentle agitation. The supernatant was removed and 3 washes of the synsorbs were performed, using a 0.15% solution of BSA in PBS. Each synsorb was tested in triplicate. The bound radioactivity was determined on the Beckman 8000 gamma counter using the I-125 dpm program.

4. Binding to Tumour Cells

RI, EL4 or BW5 cells, which excluded trypan blue in excess of 95% and were in the log phase of growth, were used to test the binding of the radiolabelled antibodies or lectin. Aliquots containing $2 - 10 \times 10^6$ cells were added to BSA-precoated tubes along with 0.5 ml 1.5% BSA. The supernatant solution was removed following centrifugation (500 rpm x 5 min.) and 100 μ l of radiolabelled antibody or lectin was added to the pellet. The tumour cell pellets were resuspended in the radiolabelled protein solutions and the total added radioactivity was determined before the mixtures were incubated on ice for one hour. Cells were washed (3x) with tissue culture media before the final supernatant was removed and the pellet recounted for bound radioactivity. The specificity of binding was assured by inhibition of binding in the presence of galactose in a manner similar to the N'RBC cell studies.

F. METHODS OF DETERMINING THE IN VIVO BIODISTRIBUTION OF RADIOLABELLED ANTIBODIES AND PEANUT LECTIN

1. Mouse Tumour Models

Six to eight week old male mice of the CBA/CAJ and C57-black strains, weighting generally between 20 and 25 grams, were purchased from the Small Animal Program, University of Alberta. They were housed in groups of 5 or 6 animals per cage and maintained on standard laboratory chow (Wayne^R Lab-Blox, Chicago, Il.) and tap water (ad libitum).

Tumour cells used for inoculation were determined to be greater than 95% viable by the trypan blue exclusion test. Following determination of the cell concentration, the suspension was centrifuged at 500 rpm for 5 minutes and resuspended in culture media, to the required concentration. The desired number of cells, generally $2 - 5 \times 10^5$ cells, was drawn up in syringes, in an aseptic environment, in the laminar flow hood. The mice were inoculated with a 0.1 or 0.2 ml subpannicular injection, in the dorsal region. RI lymphoma cells were used in CBA/CAJ mice and EL4 lymphosarcoma cells were used in C57/Black mice.

Tumours were allowed to grow for 10-14 days, before the animals were utilized for biodistribution or imaging studies of the radiolabelled protein. Lugol's solution (5 drops per 200 ml) was added to the drinking water commencing 2 days prior to injection of the radiolabelled product and continued until termination of the studies.

2. Biodistribution Studies in Tumour Bearing Mice

Tumour bearing mice were injected i.v., via the tail vein, with 0.1 or 0.2 ml of the radioiodinated antibodies (anti-T specific or nonspecific IgG, intact or $F(ab')_2$ fragment) and/or peanut lectin. In dual label experiments the nonspecific protein (IgG; intact or $F(ab')_2$) was labelled with I-131 and the specific protein (anti-T I or peanut lectin) was labelled with I-125). In single experiments the I-125 isotope was used.

The relative amount of radioactivity injected into each animal was determined by counting the syringes before and after injection, on a whole body counter. Three extra doses were prepared in syringes and counted on a whole body counter before and after being used to prepare 1:50 dilutions. Aliquots of 0.5 ml of these dilutions served as injection standards to correlate injected dose to the radioactivity determined for tissue samples.

The mice were etherized and then sacrificed by cardiac puncture/exsanguination at time periods such as 3, 8, 24 and 48 hours following injection. The tissues of interest were excised in their entirety, blotted free of blood and were weighed directly in tared plastic counting tubes (Amersham Spectra^R vials). The remaining carcass, tail (injection site), a portion of the trachea with the thyroid, were also placed in counting tubes but not weighed.

Samples, along with the diluted injection standards, were assayed for radioactivity on a programmable automated NaI gamma well counter (Beckman 8000 gamma counter). In studies involving only an ¹²⁵I-labelled protein, the coincidence method was used for absolute determination of ¹²⁵I radioactivity. In dual label experiments, a spillover correction was employed to correct for counts occurring in the ¹²⁵I window due to ¹³¹I radioactivity.

The percentage of radioactivity per gram tissue or entire organ was calculated on the basis of the injected activity and as a percentage of the activity remaining in

the body at time of dissection. Tissue ratios such as tumour:blood and tumour:muscle were calculated on a per weight basis.

a) ^{125}I -PNA in CBA/CAJ Mice with RI Tumours

Male CBA/CAJ mice of about 25 g weight were inoculated s.p. in the right flank with 5×10^5 RI lymphoma cells. Eleven to fifteen days following inoculation, the tumour-bearing mice received a 0.2 ml caudal i.v. injection of 50 kBq ^{125}I -PNA (185 kBq/ μg). Between 6 and 8 mice were sacrificed at each time period of 3, 8, 24 and 48 hours following injection and analyzed for I-125 radioactivity as described earlier.

b) Paired-label Studies

i) Anti-T vs. IgG in CBA/CAJ mice with RI tumours

CBA/CAJ mice were inoculated s.p. with 6×10^5 RI cells over the right flank, 9 days prior to use in biodistribution studies. Anti-T antibodies were labelled, as described, to a specific activity of 2 MBq/ μg with I-125 and concentrated on an Amicon Concentrator. TCA precipitation of the protein solution revealed that $91.5 \pm 1.3\%$ of the radioactivity was protein bound. Nonspecific IgG antibodies were labelled to a specific activity of 7.5 MBq/ μg with I-131 and purified on a Sephadex PD-10 column. 99.11 \pm 0.06% of an aliquot of the desalted preparation was precipitated during TCA analysis. Mice were

injected i.v. with a simultaneous injection of 10 MBq anti-T and 8 MBq IgG. Animals were dissected at 2, 4, 8, 24 and 48 hours following injection. Tissue samples were analyzed for ^{125}I and ^{131}I radioactivity, using a spillover correction program on the Beckman 8000.

ii) PNA vs F(ab')₂ in CBA/CAJ mice with RI tumours and C57-BL mice with EL4 tumours

CBA/CAJ mice were inoculated with 5×10^5 RI cells and C57 black mice were inoculated with 6×10^5 EL4 cells, 10 - 12 days prior to biodistribution studies. A 0.2 ml i.v. tail injection containing 3 MBq ^{125}I -PNA (165 kBq/ μg) and 3.5 MBq ^{131}I -F(ab')₂ IgG fragment (150 kBq/ μg) was given to the tumour bearing mice. TCA precipitation and ITLC (85% methanol in water) analysis revealed that $96.12 \pm 1.32\%$ and $95.92 \pm 1.49\%$ of the I-131 and I-125, respectively, was protein bound. Four to six mice were dissected at each of the time periods of 24 and 48 hours for the biodistribution studies in CBA/CAJ - RI bearing and C57/Bl-EL4 bearing mice. Additional CBA/CAJ mice were dissected at 8 and 72 hours. The samples and standards were analyzed for I-131 and I-125 radioactivity as described earlier.

3. Analysis of Plasma Samples from Biodistribution Studies

When the biodistribution study blood samples were taken by cardiac puncture, an aliquot was also placed in 1.5 ml

Eppendorf^R centrifuge tubes. Following centrifugation (12,800 rpm x 2 min.) a 200 μ l aliquot of plasma was transferred to another Eppendorf centrifuge tube and 200 μ l of 20% trichloroacetic acid (TCA), was added, mixed on a vortex-vortex-mixer and centrifuged. The radioactivity in the precipitated protein and supernatant was determined with a Beckman 8000 gamma counter.

4. Whole Body Gamma Camera Imaging

CBA/CAJ mice were injected with 330 kBq 125 I anti-T intact IgG or 350 kBq 125 I-PNA and anaesthetized with Nembutal^R 10 minutes prior to imaging. A PhoGamma IV (Searle) with a pinhole collimator was calibrated with an 125 I standard. Each mouse was secured with masking tape to a positioning board and serial images of the posterior view (15,000 - 20,000 counts) were obtained at time periods between 3 and 72 hours post-injection of the radiolabelled proteins.

RESULTS AND DISCUSSION

A. TUMOUR CELL LINES AND ANIMAL TUMOUR MODEL

In order to study the in vivo localizing capability of T antigen-avid proteins, it was desirable to have an appropriate animal tumour model. Reproducible moderate size tumours, which are rapidly produced with a vascular supply similar to naturally occurring tumours, would provide an ideal tumour model, if the cells expressed the T antigen²³⁵.

A number of mouse tumour cell lines were tested by Dr. J. Bray, WW Cross Cancer Institute, by direct and indirect immunofluorescence, for the binding of T antigen-avid reagents. An RI lymphoma mouse cell line was shown to bind T-affinity purified human and rabbit antibody and peanut lectin.

The RI radiation-induced lymphatic leukemia was initially isolated from CBA(H-2^K) mice by Dr. H.B. Hewitt in 1959²³⁶ and has been passaged in mice as the ascitic form and grown in vitro as a tissue culture cell line by several workers²³⁶⁻²⁴⁰. This cell line grows as a suspension in vitro, thereby facilitating cell quantitation, animal inoculation and cell viability determinations. Since the cells grow singly in suspension (although with a slight tendency to clump) trypsin treatment of the cells was not required prior to mice inoculation. This was an important parameter for the animal tumour model, since trypsin is known to cleave off cell surface glycoproteins, such as the T antigen⁴³ and is cell toxic²⁴¹.

As a radiation-induced tumour, this lymphoma may provide a more suitable animal model for spontaneously T antigen-bearing cancers in humans than a chemically or virally induced tumour. Those animal tumours which are induced by carcinogens or viruses, often express many neo-antigens and this results in an artifactual immunogenicity and antigenicity, not usually seen in spontaneous and most radiation-induced tumours²⁴¹⁻²⁴².

The tumour cell line had been passaged for an extended period of time as an in vitro tissue culture and this may possibly allow selection of cells adapted for in vitro growth, rather than growth in animals. To determine if the RI leukemic cells would grow in vivo, as a solid subpannicular tumour which could be readily imaged and dissected, CBA/CAJ mice were inoculated with 1×10^5 , 2×10^5 , 5×10^5 and 10×10^5 viable tumour cells. Solid, palpable tumours were evident, for all doses, 5 to 8 days following injection, with the largest doses generally resulting in tumours of larger masses. After 9-13 days in vivo growth, tumour masses ranged from 150 mg to 1500 mg. Larger tumours tended to have necrotic centres and showed wide ranges of degree of vascularization. In further experiments, the CBA/CAJ mouse-RI tumour model was used 9-12 days following the s.p. inoculation of 2×10^5 to 6×10^5 viable tumour cells.

Another murine mouse tumour line, utilized for in vivo and in vitro studies, was the EL4 lymphoid tumour. Gorer²⁴³ isolated it in 1945 from C57 black (H-2^b)

mice, following treatment with 9:10 dimethyl 1:2 benzanthrene and it has been maintained by tissue culture and serial i.p. and s.p. transmissions. The EL4 cells were found to not bind T antigen-avid antibodies and lectins (Bray, unpublished) and this cell line was used as a control for both in vivo and in vitro studies with the RI cells. Mice, of the strain C57/B1, were inoculated with 5×10^5 cells, and tumours, with gross appearances similar to RI tumours were ready for biodistribution studies 10-12 days later.

The BW-5147 is a lymphocytic leukemia, which spontaneously originated in an AKR mouse (H-2^K) in 1954 in the Jackson Laboratory²⁴⁴. This tumour cell line, which has been maintained by i.p. and s.p. passages and in vitro culturing, was used as a non-T antigen expressing control (Bray, unpublished) for the RI cells. Attempts to grow this tumour in CBA mice (with the same major histocompatibility classification as AKR) were unsuccessful, probably due to minor histocompatibility differences.

B. PRELIMINARY STUDIES WITH ANTI-T AND NONSPECIFIC IgG ANTIBODIES

1. Modifications of the Radioiodination Procedures

It was desirable to develop an efficient labelling procedure for ¹²⁵I-anti-T which produced a radioiodinated antibody that retained a high affinity for T antigen-containing substances. Bray and coworkers²⁴⁵ reported that ¹²⁵I-anti-T, labelled with a protected binding site, showed

a greater biological T-binding affinity than if labelled directly. A labelling method, in which the antibody was bound to the synthetic T immunoadsorbent (ChemBioMed, Ltd.) prior to iodination, was therefore chosen.

The initial labelling procedure, involved preincubation of 100 µg of anti-T antibody with 10 mg T sorb in order to protect the binding site. The reaction mixture, in 500 µl borate buffer, was gently agitated for 2 hours at 4°C within a 3 ml Reacti-Vial. A centrifugation step (5 min. at 800 rpm) was followed by 6 washes (2 ml borate buffer, pH 7.2) in order to remove antibody which was not T-sorb-bound. The protected antibody was mixed with 15-20 MBq Na¹²⁵I in 1 ml borate buffer for 30 seconds, followed by the addition of 6.86 ng ICl in 100 µl 2N NaCl. The vial was agitated on a vortex mixer for 2 minutes and the reaction was stopped by the addition of 100 µl fixing solution (0.016 M Na₂S₂O₃ and 0.012 M KI). Unreacted iodine was removed by withdrawal of the supernatant and 4 subsequent 2 ml washes of the T-sorb with PBS. The radioiodinated protein was eluted from the T-sorb with 500 µl of 1% NH₄OH and then adjusted to pH 7.2 with KH₂PO₄ (16.5% solution).

This procedure was systematically modified in subsequent experiments to optimize the retention of biological activity of the radioiodinated product. Most of the modifications were made to decrease mechanical or chemical damage to the protein during the T-sorb incubation and iodination steps. Mechanical or chemical damage may alter the quaternary or

tertiary structure of the protein, which may lead to changes in the binding affinity of the protein for T antigen-containing substances.

Centrifugation of the reaction mixture to isolate the T-sorb-protein complex was found to be unnecessary. The immunoadsorbent matrix (silylated, calcined, diatomaceous earth) was of sufficiently high density to separate efficiently by gravity alone. Six washes were initially performed on the antibody-T sorb complex prior to labelling. The number of washes was reduced to three, since the rabbit anti-T had already been purified on T-sorb. The vigorous vortexing, initially carried out during iodination procedures, was changed to gentle hand agitation.

The borate buffer was replaced by a phosphate buffer because it was suspected that the borate ion may interact with either the antibody or the T-antigen immunodeterminant disaccharide. Complexes of the type in Fig. 6 can be formed with the 4- and 6- hydroxyls and all vicinal cis-hydroxyls of pyranoside hexosides²⁴⁶. The borate ion has been shown to prevent specific binding between some anti-carbohydrate antibodies or lectins and various polysaccharides²⁴⁷. Antibodies also contain a small percentage of

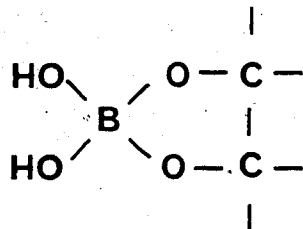


Figure 6 . Carbohydrate-Borate Ion Complex

carbohydrate (3-13%)⁹⁹, which may interact with the borate buffer. Parallel synthesis of ¹²⁵I-anti-T, using either borate or phosphate buffer, resulted in similar labelling efficiencies (40-43%) and desorption capabilities of the anti-T (47-51%). Thus subsequent iodinations were conducted in phosphate buffer.

The relatively high ICl/protein ratios, used initially to ensure high labelling efficiencies, exposed the protein to higher concentrations of the oxidizing agent than were necessary. The possible subsequent chemical damage and/or a high incorporation of iodine atoms into the protein, may change the biological activity of the antibody²⁰⁸. The molar ratio of ICl was decreased four-fold, so that the maximum iodine incorporation would be about 1.5 atoms per antibody molecule, based on 100% chemical yield.

The chemical exposure of the protein was also reduced by elimination of the reducing agent (sodium metabisulfite). Since the antibody is immobilized on the T-sorb particles, the oxidizing reagent and free I⁺ and were simply removed with the supernatant and four washes of the T-sorb protein complex. Duplicate reactions, in which the reducing step was omitted in one of the reactions, revealed that a higher hemagglutination titre to N'RBC was obtained for the antibodies labelled without reduction. The binding of the nonfixed antibody preparation was 4.7% to N'RBC and only 0.3% to control RBC.

Once the antibody is radioiodinated on the T-sorb, a desorption agent is required to elute the labelled antibody from the immunoadsorbent. A number of desorption agents were considered. Although specific desorption could be achieved with galactose, the galactose would then occupy the combining site on the anti-T molecules and preempt subsequent binding. Extensive manipulation, to remove galactose from the radioactively labelled protein in procedures such as dialysis may be undesirable. Such techniques may result in an increase in the radiation dose to the handler and lead to greater nonspecific losses of the protein by surface adherence. Antibody-antigen complexes can also be dissociated nonspecifically by conditions which lead to reversible conformational changes of the antibody molecules. Nonspecific desorption has been initiated by extreme pH changes, or high concentrations of chaotropic ions, or urea, or guanidine hydrochloride²⁴⁸.

In the methodology developed by ChemBioMed, an increased pH, with the use of 1% NH_4OH , was utilized⁸⁹ to elute antibody from the T-sorb. To evaluate the effects of various concentrations of NH_4OH for antibody desorption, two labelling experiments were carried out in which aliquots of the labelled antibody-T sorb complexes were desorbed with either 0.5%, 1%, or 2% NH_4OH . The desorption capability was found to be highest for the 2% NH_4OH , which eluted 79-85% of the T-sorb-associated radioactivity, as compared to 70-74% and 54-57% with the 1% and 0.5% concentrations,

respectively. The radioiodinated antibody preparations, eluted by all three NH_4OH concentrations, showed similar binding to T-sorb ($\sim 70\%$) and N'RBC ($\sim 5\%$). A 2% NH_4OH concentration was therefore selected, due to its greater desorption capabilities. Other methods of desorption, including 0.1 M KCl pH 2.2 for 30 seconds or 3 minutes, 0.4 M borate buffer for 1 minute, or 1% acetic acid for 1 minute, were all found to be less effective than 2% NH_4OH in eluting the labelled antibody from the T-sorb.

Nonspecific adsorption of the antibody to contact surfaces was a very significant problem throughout any of its manipulations. Losses as high as 25% on Falcon^R tubes, 85% in an Amicon^R B-15 concentrator, and 50% on a Sephadex PD-10 column, were observed. It was therefore absolutely necessary to precoat every surface, that would come in contact with the antibody, with a 1% BSA solution to decrease the nonspecific adsorption losses.

Various methods of concentrating the protein solution and removing free iodide were evaluated using an unpurified radiolabelled IgG preparation, containing 50% free iodide and 50% labelled protein (based on ITLC analysis with 85% aqueous methanol). Lyphogel^R, which excludes macromolecules larger than 20,000 MW and absorbs five times its weight in water and salts, was found to be unsatisfactory. Although the protein became concentrated, only 54% of the added radioactivity was recovered and ITLC analysis of this solution revealed that 50% of the

recovered radioactivity was still in the form of free iodide. A BSA preincubation step was necessary to prevent protein absorption losses (which were as high as 65%) and a wash of the pellets was necessary to allow retrieval of the protein solution. Selective removal of water, but not free I^- , was also found to be a problem with the Amicon B-15 macrosolute concentrator. Following 4 to 6 fold concentration on an Amicon^R concentrator, free iodide levels generally remained at 70-95% of the initial levels. When an ion exchange resin column (Cl^- form of Dowex-AG, Type 21K, Mesh 50-100) was used to purify an IgG preparation, the free iodide was reduced from 32% to 4% but only 21% of the protein was recovered from the BSA precoated column. The most efficient way of removing free iodide from the antibody, without significant loss of protein, was desalting using gel filtration chromatography.

A small percentage (6-10%) of free iodide was usually observed in the desorbed protein, even though immobilization on T sorb should theoretically allow total removal of free iodide and reactants, before desorption. Analysis of the total radioactivity in the supernatant and 4 subsequent PBS washes of the antibody-T sorb complex indicated that only 2.5% of the total recovered radioactivity was present in the final two washes. Therefore insufficient washings of the T-sorb was unlikely to be the source of free iodide.

A blank labelling experiment, in which phosphate buffer replaced the protein, indicated that 25% of the total

radioactivity used in the reaction remained associated with the T-sorb and reacti-vial after washing the T-sorb. The NH_4OH desorption process then eluted 28% of the radioactivity on the T-sorb and reacti-vial. The source of free iodide in the antibody preparations, therefore appeared to be nonspecific adsorption and desorption of I^- from the reacti-vial and T-sorb. A purification technique, such as gel filtration was therefore needed to remove the 6-10% free iodide from the anti-T preparations following desorption.

The final modified labelling procedure for the Anti-T rabbit IgG and nonspecific rabbit IgG is described in sections C2a and C2b, respectively, in the Methodology. The protected T-sorb iodine monochloride iodinations of anti-T resulted in labelling efficiencies which ranged from 25-50% and the direct iodination of IgG gave iodination efficiencies of 50-80%.

2. Biodistribution Studies and In Vitro Binding

Preliminary biodistribution studies were carried out with ^{125}I -anti-T in RI lymphoma-bearing CBA/CAJ mice. It was found that the ^{125}I -labelled anti-T localized in the tumours to some extent but that prolonged, elevated blood levels maintained a high background-tissue radioactivity. Three preliminary biodistribution studies indicated that average tumour:muscle and tumour:blood ratios of radioactivity at 24 hours (total of 10 mice) were $5.9 \pm 0.5:1$ and $0.57 \pm 0.04:1$, respectively. Preliminary gamma camera

imaging analysis, of RI tumour-bearing mice injected i.v. with 300 kBq ¹²⁵I-PNA, revealed that some tumour localization was evident 4 days post-injection.

A complication, when determining the tumour localizing capabilities of an antibody or any protein preparation, is the nonspecific accumulation of macromolecules in tumours²⁴⁹. Tumours often have a larger extravascular-extracellular space, greater blood vessel permeability and less efficient lymphatic drainage than found in normal tissue¹⁹. Therefore, most radiolabelled proteins localize to some extent in tumours. The use of double isotopic labels, a technique first introduced by Pressman et al.²⁵⁰, is helpful in distinguishing between specific and nonspecific antibody localization in tumour and other tissues. Specific anti-tumour antibodies are labelled with one isotope of iodine, eg. ¹²⁵I, and normal control globulins are labelled with another, such as ¹³¹I. Simultaneous injection of the two preparations allows evaluation of the tumour for localization of specific antibody and control nonspecific protein²⁵⁰.

This paired-label technique was therefore utilized to determine the specificity of anti-T tumour localization. Mice bearing RI tumours were simultaneously injected with 10 MBq ¹²⁵I- anti-T and 8 MBq ¹³¹I-IgG. The localization of the antibodies in tissue samples was subsequently determined by differential radioactive analysis of the two isotopes present. Six mice were dissected at each time period of 2, 4, 8, 24 and 48 hours. The radioactivity, determined

for ^{125}I -anti-T, was corrected for the spillover of ^{131}I counts occurring in the ^{125}I window. Biodistribution data was calculated as a percentage of the injected dose per gram of wet tissue or per intact organ and are presented in Table 12 and Table 13 for the 8, 24 and 48 hour time periods. Although not assayed in this particular biodistribution study, preliminary studies indicated less than 1% of the injected dose was contained in the lung at 24 hours.

The ratio of radioactivity for tissue: blood and tissue: muscle, are also presented in Appendix 1 and were calculated on a gram to gram basis. These ratios show the relative concentrations in the various tissues and may give an indication of the potential usefulness in imaging studies.

The best imaging results, in most antibody tumour localizing studies, have usually occurred several days post-injection, when the background blood activity has diminished considerably²⁴. Otherwise, computerized subtraction of blood-pool background activity is often employed²⁵⁻²⁷.

A high blood background radioactivity was also found to complicate this study. From the organs dissected, and analyzed for ^{125}I -anti-T, only the kidneys and tumour were found to have tissue: blood ratios of radioactivity larger than 0.30:1 at any of the time periods evaluated. The ^{125}I -anti-T tumour: muscle ratio was 7.4:1 at 8 hours, 5.4:1 at 24 hours and 4.9:1 at 48 hours. The tumour: blood ratio remained at 0.5:1 for both the 24 and 48 hour time periods. Preliminary biodistribution studies revealed tumour: blood

COMPARATIVE TISSUE BIODISTRIBUTION OF
OF I-125 ANTI-T AND I-131 NORMAL GAMMA GLOBULIN
IN R_J LYMPHOMA-BEARING CBA/CAJ MICE

TABLE 12 (PERCENT DOSE PER GRAM TISSUE)

	%DOSE/GRAM STAN.DEV.	24HR(N=6)		48HR(N=6)		8HR(N=6)		24HR(N=6)		48HR(N=6)	
		T-AB	IGG	T-AB	IGG	T-AB	IGG	T-AB	IGG	T-AB	IGG
TUMOUR	4.67 1.22	7.31 1.93	4.08 0.81	7.14 1.71	2.74 0.77	5.08 1.94	1.91 1.20	3.01 1.94	1.51 0.74	2.60 1.24	3.60 1.66
BLOOD	11.65 2.30	20.43 4.72	8.10 0.79	15.88 2.01	5.50 0.92	11.11 2.38	16.66 3.28	29.21 6.75	11.58 1.13	22.71 2.88	15.89 3.41
LIVER	3.22 0.62	4.21 1.03	2.49 0.22	3.69 0.37	1.62 0.30	2.41 0.64	4.16 0.74	5.42 1.21	2.93 0.22	4.34 0.39	2.87 0.80
SPLEEN	3.01 0.80	4.57 1.26	2.17 0.23	3.78 0.52	1.64 0.40	2.92 0.75	0.32 0.12	0.49 0.18	0.24 0.05	0.42 0.07	0.30 0.04
KIDNEY	4.59 1.07	6.22 1.69	3.95 0.67	5.74 1.08	2.46 0.44	3.71 0.86	1.59 0.35	2.15 0.52	1.24 0.17	1.81 0.32	1.25 0.38
MUSCLE	0.66 0.22	1.02 0.33	0.78 0.17	1.37 0.29	0.63 0.27	1.16 0.38					

TABLE 13

	%DOSE/ORG STAN.DEV.		%DOSE/ORG STAN.DEV.		%DOSE/ORG STAN.DEV.		%DOSE/ORG STAN.DEV.		%DOSE/ORG STAN.DEV.	
	T-AB	IGG	T-AB	IGG	T-AB	IGG	T-AB	IGG	T-AB	IGG
TUMOUR	1.91	3.01	1.51	2.60	2.06	3.60	1.20	1.94	1.24	1.00
BLOOD	16.66	29.21	11.58	22.71	7.86	15.89	3.28	6.75	1.13	2.88
LIVER	4.16	5.42	2.93	4.34	1.91	2.87	0.74	1.21	0.22	0.39
SPLEEN	0.32	0.49	0.24	0.42	0.17	0.30	0.12	0.18	0.05	0.03
KIDNEY	1.59	2.15	1.24	1.81	0.82	1.25	0.35	0.52	0.17	0.32
MUSCLE										

FOOTNOTE: T-AB=ANTI-T ANTIBODIES; IGG= NONSPECIFIC NORMAL GAMMA GLOBULIN

ratios at 24 hours of 0.4:1 and 0.15:1 for lymph nodes and thymus respectively.

The IgG preparation was found to have a greater whole body and tissue retention than the specific anti-T. The percentage of injected dose of ^{131}I -IgG, per gram of tissue, was found to be 1.5-2 fold higher in most tissues as compared to anti-T. Similar findings were observed by Searle and coworkers²⁹ when they compared the biodistribution of non-specific rabbit IgG and anti-HCG (antibodies to human chorionic gonadotrophin), in nude mice bearing human choriocarcinoma xenografts. At five days post-injection, blood levels were an average of 4 fold higher for the non-specific antibody than for specific anti-HCG. These differences were not observed in non-tumour bearing mice²⁹.

Although the absolute tissue retention of the IgG was elevated, relative tissue ratios such as tumour:muscle and tumour:blood were very similar to those observed for anti-T. At 24 hours the tumour:muscle ratio was 5.1:1 and the tumour:blood ratio was 0.45:1 for the ^{131}I -IgG. Similar tumour:blood ratios have been observed when radiolabelled non-specific IgG was injected in tumour bearing rats²⁵¹.

An informative manner for the analysis of the specificity of antibody localization, is to calculate the specificity or localization index^{28, 252, 253}. The ratios of relative concentrations such as tissue:blood, obtained for the specific antibody are divided by the corresponding factor, calculated independently for the control protein²⁸.

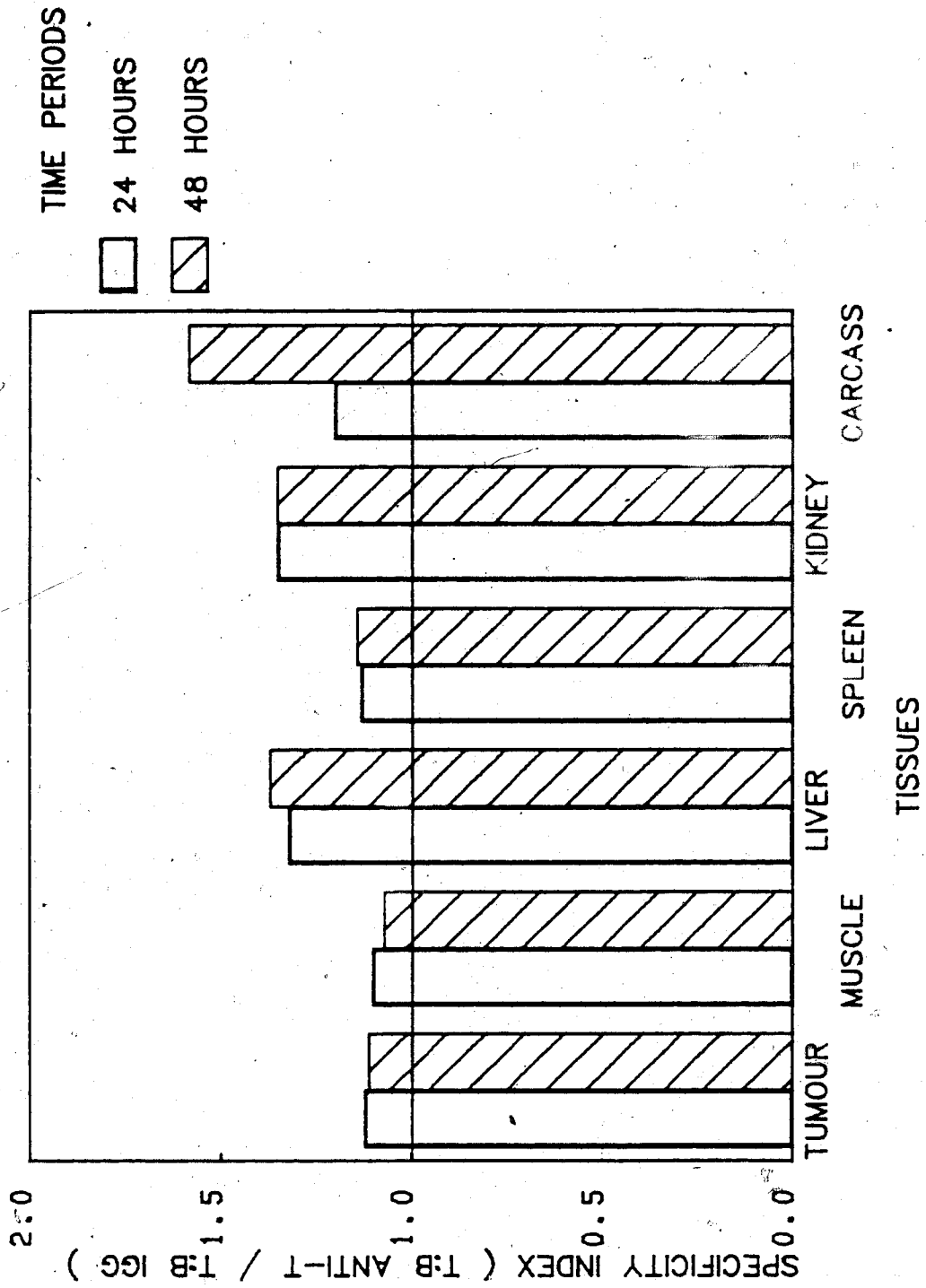
The specific:nonspecific ratio can be standardized by ratios in the blood²⁸, injected doses²⁵², liver^{29, 253}, lung²⁹ or muscle²⁹.

In this study, the blood was chosen to analyze the data, since 1) poor blood clearance is one of the major complications in antibody studies²⁷ and 2) nonspecific macromolecular tumour localization is primarily due to a changed vasculature in the tumour tissue¹⁹. The ratios were therefore calculated using the percentage of injected dose per gram of blood or tissue in the following equation.

$$\text{specificity index} = \frac{\text{tissue:blood anti-T-IgG}}{\text{tissue:blood nonspecific IgG}}$$

When this ratio is much greater than one for the tumour but not other tissues, it indicates that the increased antibody radioactivity in the tumour is probably due to specific tumour tissue binding in addition to hypervascularization and interstitial fluid accumulation in the tumour. The specificity indices calculated for various tissues are shown in the histogram, Fig. 7. The specificity index of the radioiodinated anti-T for the tumour was 1.11 to 1.13 at the time periods of 8 to 48 hours and was not found to be significantly different from the remaining carcass or other tissues. Primus et al.¹⁸ undertook studies with affinity purified anti-CEA, in hamsters bearing GW-39 tumours and analyzed the data with a specificity index, standardized by the injected dose, instead of blood. Although most other tissues had a specificity index of 0.96 to 1.14 at 48 hours

FIG. 7
ANTI-T SPECIFICITY INDICES
AT 24 AND 48 HOURS



the value from the tumour data was 2.34¹⁸. This indicated localization in the tumour, in which nonspecific accumulation could only account for about 43% of the tumour retention. In the anti-T/IgG study, approximately 90% of the localization in the tumour, and most other tissues, is probably due to nonspecific localization. This seems to indicate that the anti-T is not specifically retained by interaction with the T antigen on the RI cells. Metabolism of the antibody may be occurring before it is fixed on the cell surface, since the tumour receives such a small proportion of the cardiac output¹⁸.

The rabbit anti-T immunoglobulins used in these studies perhaps did not have a high enough specificity for the RI tumour cell antigens. Heterogeneous antibodies, even following affinity purification, generally only contain a maximum of 15% specific antibody molecules²⁵⁴. Perhaps the binding affinity was not sufficient enough, so that any specific localization was masked by nonspecific macromolecular localization. Results of the in vitro binding are displayed in Table 14 and indicate the anti-T bound relatively avidly to the T immunoadsorbent, but the binding was not extremely high to N'RBC (which by definition of the T antigen, contains a large number of T antigen receptors)¹³¹. The in vitro tumour-cell binding did not indicate a high specificity of the radiolabelled anti-T for antigens expressed on the RI cells.

Table 14

In Vitro Binding of Anti-T and Normal IgG*

	Anti-T	Normal IgG
Binding to T-sorb (specific),	45 - 85% (n=8)	0.8 - 0.9% (n=4)
Binding to Synsorb (control)	2.5 - 10% (n=4)	2.1 - 2.2% (n=4)
Binding to N'RBC (specific)	3 - 8.2% (n=4)	0.20 - 0.22% (n=2)
Binding to RBC (control)	0.3 - 3.3% (n=4)	0.28 - 0.3% (n=2)
Binding to RI (specific)	1%** (n=1)	0.9%** (n=1)
Binding to EL4 (control)	0.6%** (n=1)	0.8%** (n=1)

- * - Anti-T and IgG were radiolabelled with I-125 and I-131, respectively.
 - Values are expressed as percentage of added radioactivity bound to ligands following incubation and subsequent washings.
 - The number of independent determinations is given in parenthesis.
- ** - Determined by Dr. J. Bray, WW Cross Cancer Institute.

The anti-T was prepared by injecting rabbits with the synthetic β -D-Gal(1 \rightarrow 3) α -D-GalNAc conjugated by an 8 carbon linking chain to carrier BSA molecules. Antibodies, which were specific for the linking arm or any of the many antigenic sites present on a BSA molecule, could be produced along with the antibodies specific for the T disaccharide. Affinity purification on the T immunoabsorbent should remove anti-BSA antibodies but antibodies specific for the linking carbon chain would still be present in the "purified" antibody preparation. Therefore, only a proportion of these "anti-T" antibodies would also have a high affinity for the natural antigen, exposed on the cell surface.

It was desirable to carry on these studies with a homogeneous monoclonal antibody preparation with a very high specificity for the T antigen. However, due to the sophisticated techniques involved in preparing a monoclonal antibody with a highly avid and specific binding ability, a source of monoclonal anti-T was not available to continue the antibody studies at the time.

Another T antigen-avid protein, the peanut lectin, was available as a homogeneous, affinity-purified protein. Initial in vitro binding studies indicated high binding affinities for not only the T immunoabsorbent but also membrane-bound T specificities in N'RBC and RI tumour cells.

Therefore, further studies were carried out on the peanut lectin as a radiolabelled T-avid protein for the

radioimmuno-detection of tumours bearing the T specific determinant. These studies form the major portion of the experimental investigations reported in this thesis.

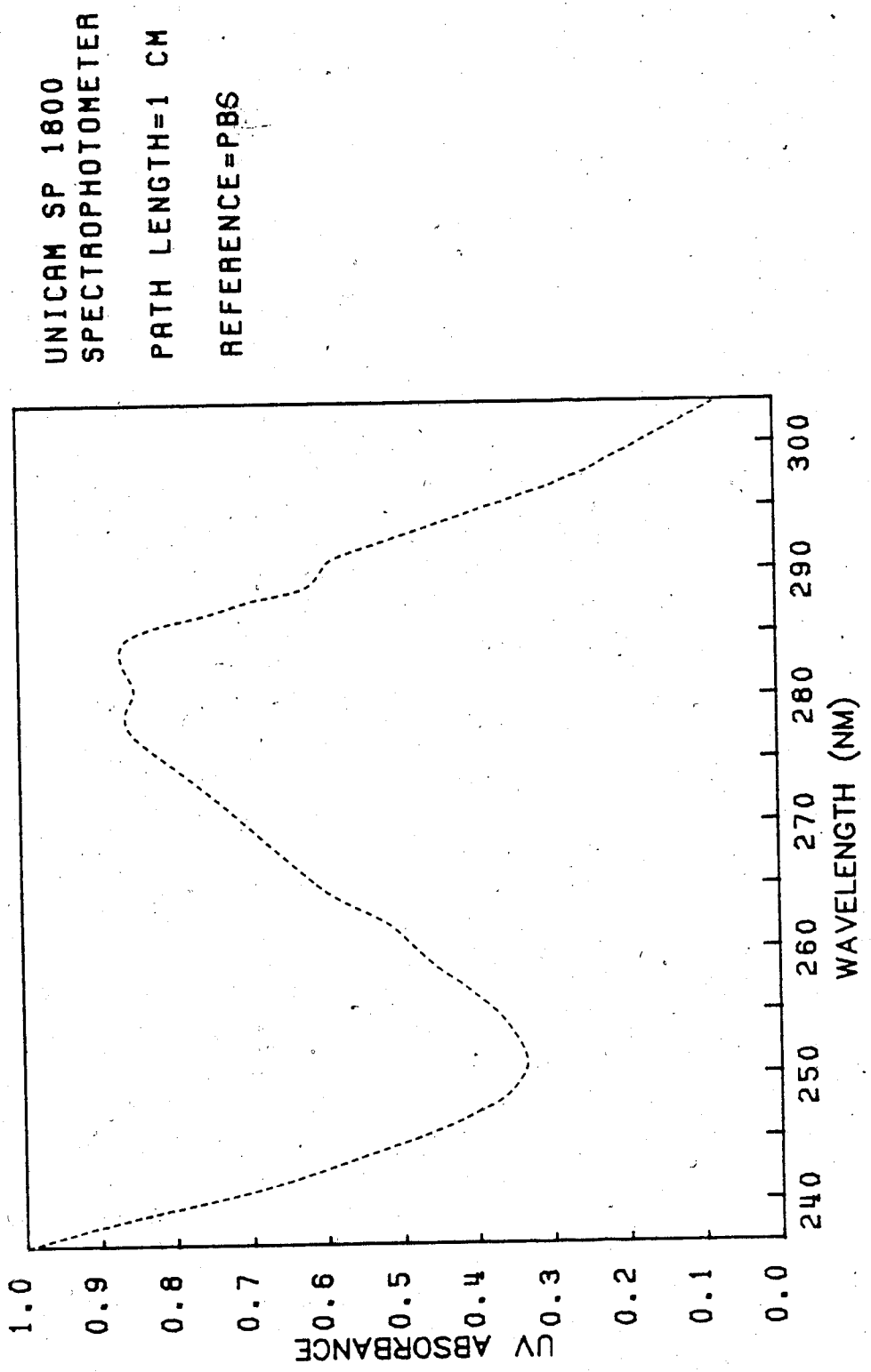
C. PEANUT LECTIN

1. Reconstitution and UV Spectroscopic Analysis of PNA

The peanut lectin, utilized in in vitro and in vivo studies, was obtained from EY Laboratories (San Mateo, California) and the product specifications indicated a single band by polyacrylamide-disc gel electrophoresis. The hemagglutination titre, of the PNA lot obtained, was 1:2048 to neuraminidase-treated red blood cells. The protein was kept frozen in the lyophilized form until portions of 3 to 5 mg were reconstituted with phosphate buffered saline, pH 7.4 (PBS). The reconstituted protein was passed through a sterile 0.22 μ m filter and maintained under aseptic conditions at 4°C.

The reconstituted protein was analyzed by UV spectroscopy at 280 nm for both qualitative and quantitative analysis. The near UV spectral properties of PNA are shown in Fig. 8. The absorption spectra revealed a double maxima at 277 nm and 283 nm and also a small shoulder peak at 290 nm. These peaks are believed to be due to aromatic amino acid side chains such as tyrosine⁶⁹ and the UV spectrum obtained on the EY Lab PNA preparation was essentially identical to that published to other investigators^{69, 112, 255}. Quantitative increases of the peaks, in

FIG. 8
NEAR UV SPECTRA OF PEANUT LECTIN



the 270-300 nm range, occur during interactions of PNA with specific saccharides and glycoproteins^{69, 79, 112}. It has therefore been postulated that certain aromatic amino acid residues of PNA, such as tyrosine, are involved in the binding site of PNA^{69, 72, 111, 112}.

Several PNA preparations were accurately reconstituted to 1 mg/ml and the optical density was measured at 280 nm. Six separate determinations of the extinction coefficient at 280 nm resulted in a value of 8.45 ± 1.26 for the $E_{280 \text{ nm}}^{1 \text{ mg/ml}}$ of PNA. Two investigators have published values for both the molecular weight and $E_{280 \text{ nm}}^{1 \text{ mg/ml}}$ of PNA, which are at variance with one another. Lotan⁵⁹ et al. reported a molecular weight of 110,000 and a $E_{280 \text{ nm}}^{1 \text{ mg/ml}}$ of 7.7. On the other hand, Fish et al.⁶⁹ claimed that the observed extinction coefficient corresponded to the tryptophan and tyrosine content that they observed in PNA, although the amino acid composition was not actually reported. If the molecular weight determination of PNA by Fish et al.⁶⁹ of 98,000, is used to adjust the extinction coefficient of Lotan et al.⁵⁹ (initially based on a molecular weight of 110,000), an adjusted $E_{280 \text{ nm}}^{1 \text{ mg/ml}}$ of 8.8 is obtained. Likewise, if the extinction coefficient of Fish et al.⁶⁹ is adjusted according to the molecular weight of Lotan et al.⁶⁹ the extinction coefficient becomes $E_{280 \text{ nm}}^{1 \text{ mg/ml}} = 8.4$. These two values of 8.4 and 8.8 are very close to the extinction coefficient value of 8.45 which was observed experimentally

for the PNA obtained from EY laboratories. Quantitative determinations of the peanut lectin concentration were calculated using the equation $A = E_{1 \text{ cm}}^1 \text{ mg/ml} \cdot c \cdot l$ (where A = absorbance, c = concentration in mg/ml and l = path length in centimeters). The $E_{1 \text{ cm}}^1 \text{ mg/ml}$ extinction coefficient used in these determinations was the published value at 280 nm of 9.6^{69} . The reconstituted peanut lectin solutions were stored at 4° at concentrations of 1 mg/ml and used within 2 months or discarded.

2. Quality Control of Radioiodine (Na^{125}I) Solution

a) Radionuclidic purity of Na^{125}I

The reagent grade, no-carrier added radioiodine (Na^{125}I), used for labelling procedures, was tested for both radio-chemical and radionuclidic purity. A NaI(Tl) well crystal and multichannel analyzer were used to determine the energy spectrum of Na^{125}I . Two photopeaks were observed, which occurred at about 28 keV and 55 keV and the gamma spectrum observed correlated well with a published spectra of I-125^{234} . The photopeaks are due to the single emissions at 27.4 keV and 35.5 keV, and the coincidence peaks which occur at 55 and 63 keV. No peaks were observed at higher energies.

b) Radiochemical purity of Na^{125}I

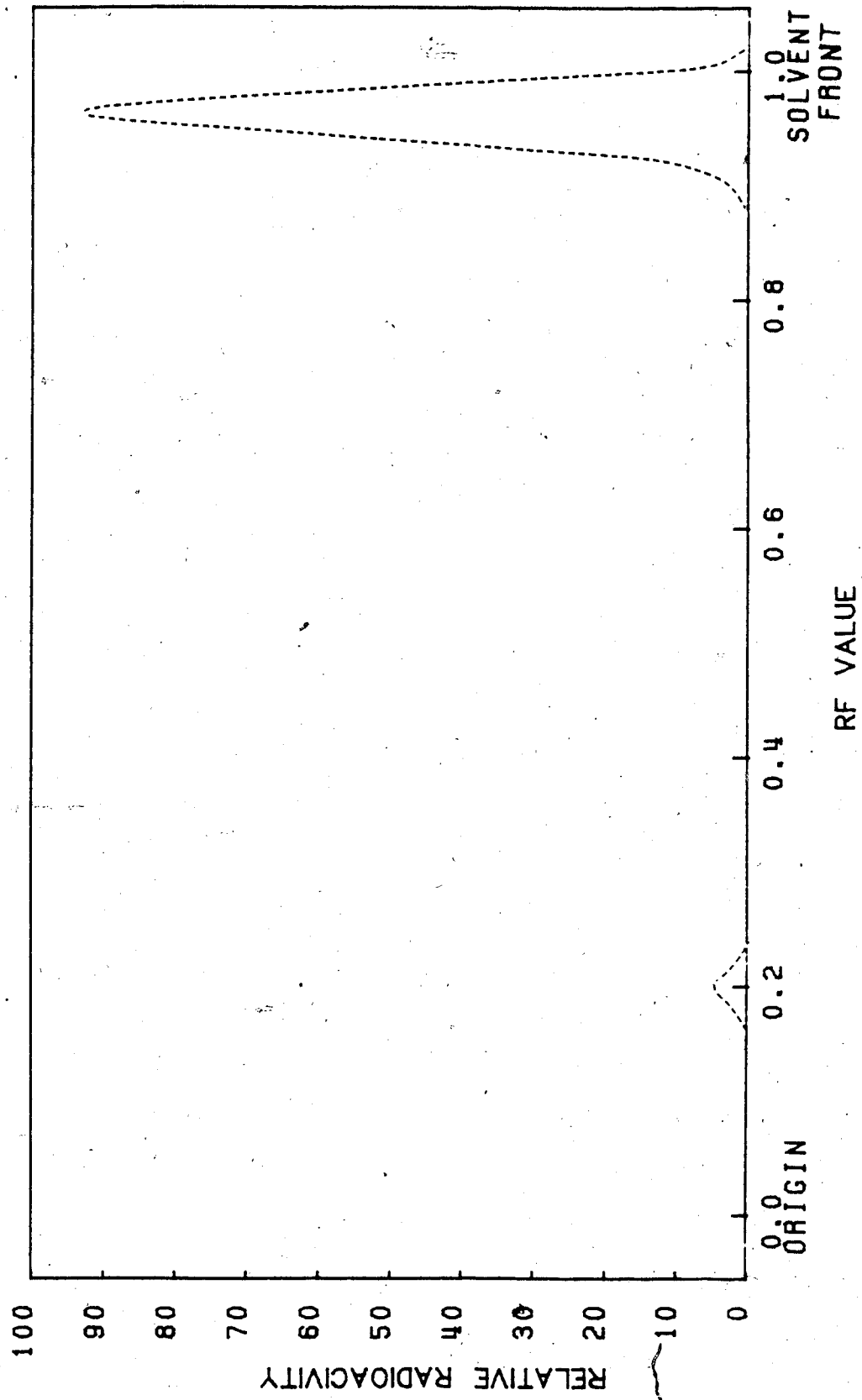
Instant thin layer chromatography (ITLC) was used to analyze the radiochemical purity of the radioiodine.

Aliquots of the Na^{125}I were spotted on silica gel ITLC strips and developed by ascending chromatography (85% methanol in water), to determine the presence of any iodate. A chromatogram scan of the developed ITLC strip is shown in Fig. 9. In this system, the I^- migrates with the solvent front, with typical R_f values of 0.9, while the radioiodate (IO_3^-) remains close to the origin with an R_f value about 0.2²⁵⁶. Analysis of the strips revealed that the radioiodide peak accounted for 97.6% of the total radioactivity. Thus this product was acceptable for radioiodination purposes.

3. Radioiodination and Radiochemical Purification of PNA

The chloramine-T method was utilized for the radioiodination of peanut lectin based on published procedures for the production of radioiodinated peanut lectin⁹⁶. The Na^{125}I was buffered initially to pH 7.4 with phosphate buffer to prevent protein damage from the alkaline pH of the radioiodine solution and to optimize the pH for the radioiodination. The ratio, on a weight basis, of chloramine-T to protein, 0.3:1, was chosen to provide a consistent labelling efficiency while minimizing exposure of the protein to extensive oxidizing conditions. The sodium metabisulfite was added in excess (twice the molar quantity of chloramine-T) in order to terminate the reaction and reduce unreacted I^+ to I^- .

FIG. 9
RADIOCHEMICAL PURITY OF RADIOIODIDE; NA 1-125



The radioiodination efficiency was assessed by three different methods; TCA precipitation, silica gel ITLC and gel filtration chromatography. Radiolabelling efficiencies were routinely determined by TCA precipitation and generally correlated well with the values determined by ITLC and for analysis of the elution profiles. The labelling efficiencies of 9 separate radioiodinations are presented in Table 15. An average labelling efficiency of $58.2 \pm 6.5\%$ was attained with a range of 50-74% based on TCA precipitation analysis. In the silica gel ITLC (85% methanol in water) analysis of the labelled protein, the unbound ^{125}I migrated at the solvent front (typical Rf values of 0.90 to 0.95) while the protein fraction remained essentially at the origin (Rf values of -0.05 to 0.15). If the samples, which were analyzed, contained a very large amount of free iodide, the estimated value of unbound radioactivity was generally higher when determined by ITLC analysis, than by TCA precipitation. For example, analysis of one radiolabelled protein/radioiodide mixture revealed 83% free iodide by ITLC and 76% free iodide by TCA precipitation. This is possibly due to non-specific trapping of the free iodide in precipitated carrier BSA protein. Addition of carrier KI during TCA precipitation may possibly help rectify this problem²⁵⁷.

Separation of unreacted radioiodine was carried out by gel filtration chromatography. Initial trials, with Sephadex 25 M in PD-10 columns, resulted in significant losses of the radiolabelled lectin on the column. This was possibly due to

Table 15

Analysis of Labelling Efficiency in
Chloramine-T Radioiodination Procedures of Peanut Lectin

Experiment	Percent Labelling Efficiency as determined by: *		
	TCA Precipitation	Gel filtration Elution Profile	Silica Gel ITCL 85% MeOH/H ₂ O
1	50.1 ± 0.3 (n=2)	48.8%	-
2	64.5 ± 4.9 (n=2)	59.8%	-
3	58.2 ± 0.8 (n=2)	56.2%	-
4	60.0 ± 0.5 (n=4)	57.9%	-
5	65.1 ± 0.3 (n=2)	64.9%	-
6	66.8 ± 0.9 (n=2)	67.2%	-
7	51.8 ± 2 (n=3)	50.3%	49.2 ± 1.3 (n=3)
8	49.8 ± 1.6 (n=3)	52.2%	49.4 ± 2.2 (n=3)
9	57.1 ± 1 (n=2)	56.1%	55.2 ± 0.8% (n=3)

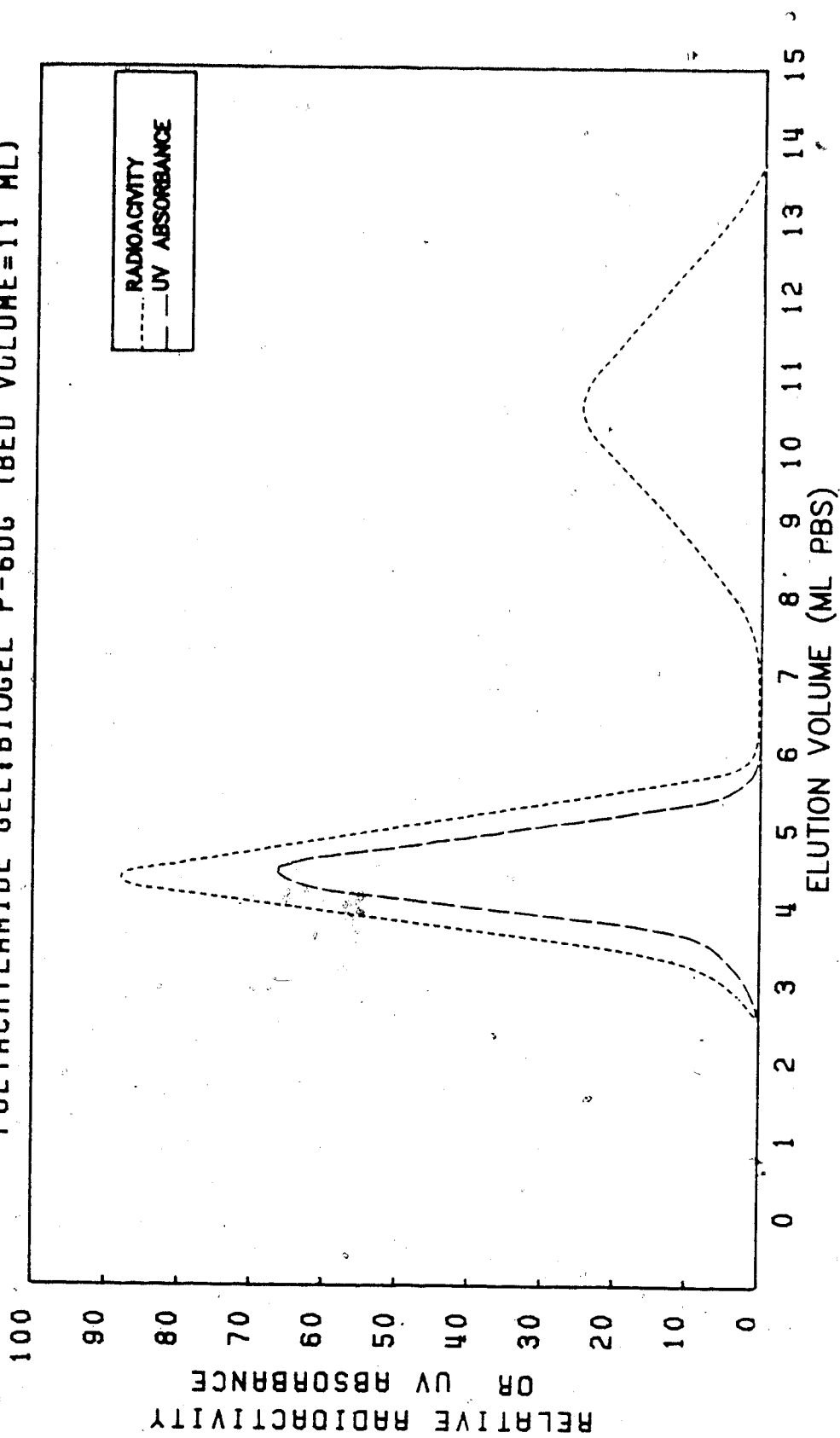
* n = number of determinations, all gel filtration calculations are based on one determination from the desalting procedure.

lectin interaction with the carbohydrate matrix of the Sephadex. Therefore a polyacrylamide gel, BioGel P-6DG, was chosen, since the media is designed for desalting procedures and exhibits low nonspecific adsorption of macromolecules. Initially, the BioGel P-6DG media was packed into 5 ml syringe barrels or emptied PD-10 columns (Pharmacia), and did not quite provide baseline resolution between the void volume (protein peak) and the salt peak (iodide). Therefore longer columns were packed (9 mm x 300 mm (Pharmacia)), with bed volumes of about 11 ml. These longer columns provided excellent baseline resolution between protein and salt peaks. A typical elution profile is illustrated in Fig. 10.

The column eluate was analyzed, in sequence, for both protein optical density (at 280 nm) and radioactivity. The protein absorbance peak was usually noted to appear at an elution volume of 2.5 ml. and fractions containing the complete absorption peak were collected in about a 3 ml volume. Generally, the first 1.5 ml of protein collected was utilized for further in vivo and in vitro studies and this fraction generally contained in excess of 80 per cent of the protein radioactivity. A further 1 to 1.5 ml was eluted at baseline radioactivity before the radioiodide salt peak occurred. Analysis of the radioactivity elution profile provided additional information on the labelling efficiency of the radioiodination, which correlated well with analysis by TCA and/or ITLC techniques. Between 92 and 99.5% of the protein-associated radioactivity added to

FIG. 10

ELUTION PROFILE OF THE GEL FILTRATION SEPARATION OF I-125 PNA
POLYACRYLAMIDE GEL: BIOGEL P-60G (BED VOLUME=11 ML)



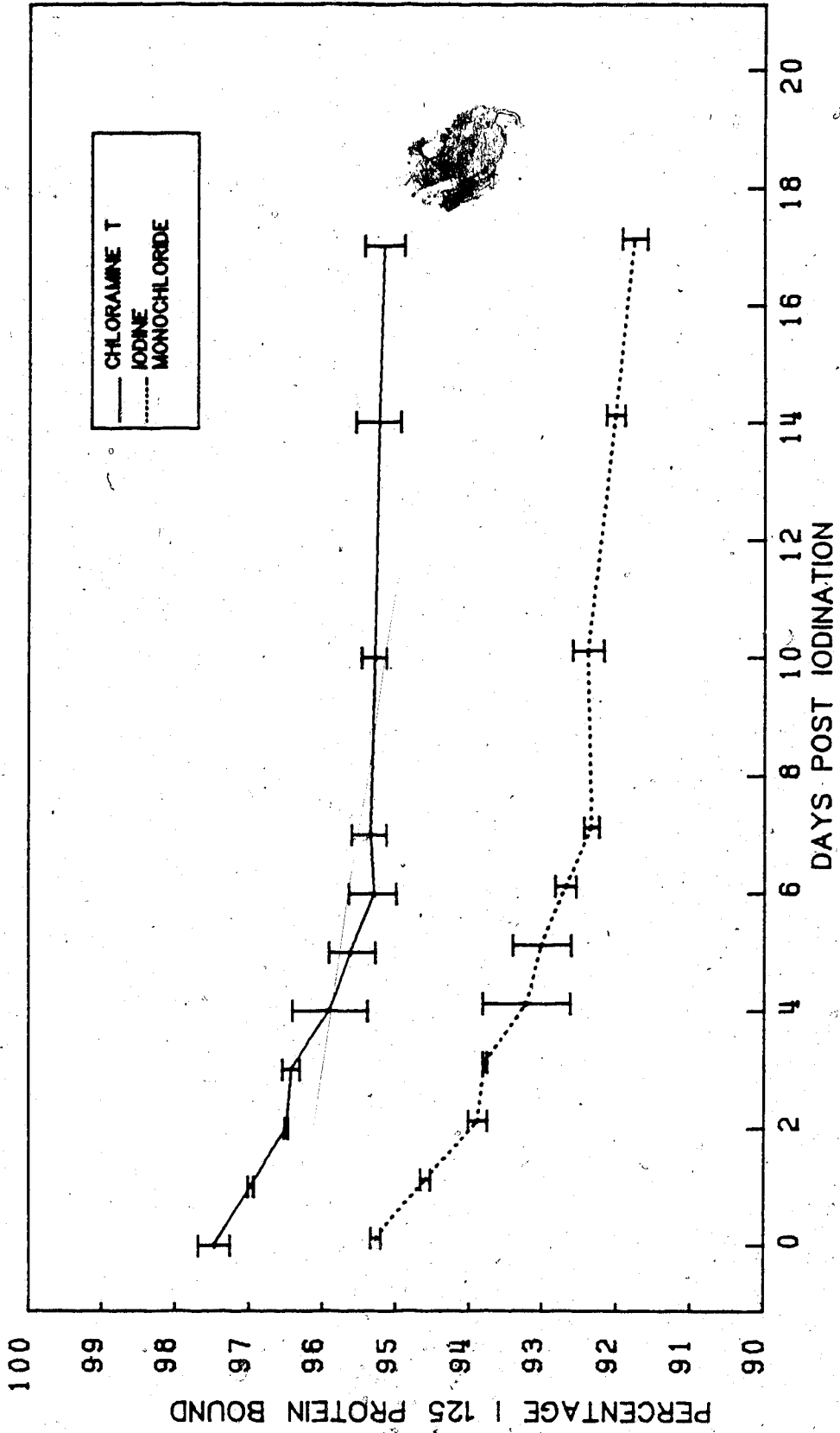
the column, was recovered in the protein peak fractions. TCA precipitation analysis of the two major radioactivity elution peaks revealed that 96-97% of the radioactivity was precipitated in the protein peak while only 5-7% of the radioactivity precipitated in the iodide peak. Therefore, the BioGel P-6DG column provided an efficient means of removing free iodide from the radiolabelled lectin and sample dilutions of the protein fractions, as a result of this desalting procedure, were usually only 3 to 5 fold.

4. Radiochemical Stability of ^{125}I -Peanut Lectin

The radiochemical stability of radioiodinated PNA was determined for ^{125}I -PNA prepared by two different radioiodination techniques; the routinely used Chloramine-T method, and also the iodine monochloride method. Both preparations were desalted separately on the same P-6DG column and the purified protein mixtures were stored at 4°C, pH 7.4 in BSA-precoated Falcon tubes.

These radioiodinated protein solutions were analyzed by TCA precipitation to determine the percentage of protein-bound radioactivity. The results of the triplicate determinations are plotted in Fig. 11 for the radioiodinated lectin solutions, labelled by the iodine-monochloride and chloramine-T methods. As illustrated in Fig. 11, the ^{125}I -PNA, labelled by the Chloramine-T method, was somewhat more stable than that produced by the ICl technique. During the initial first few days following radioiodination, the daily loss of radioiodine was more rapid than that observed after

FIG. 11
STABILITY OF I-125 PEANUT LECTIN AT 4°C
FOLLOWING CHLORAMINE T OR IODINE MONOCHLORIDE RADIOIODINATION



(EACH POINT IS MEAN ± S.D. OF 3 DETERMINATIONS)

the first week. At two weeks, 2.2 and 3.3% of the radioiodide had hydrolyzed from the Chloramine-T- and ICl- radioiodinated PNA, respectively and about 65% of this hydrolysis had already occurred in the initial 4 days.

Overall, the rate of hydrolysis of radioiodine from ^{125}I -PNA appears to be relatively slow, when the radio-labelled PNA is stored at 4°C , and a pH of 7.4.

5. In Vitro Binding Studies of ^{125}I -PNA

The biological binding affinity of radiolabelled peanut lectin was determined by various in vitro studies. The presence of a large number of Thomsen-Friedenreich receptors on neuraminidase-treated red blood cells, N'RBC, makes these cells extremely useful ligands to test the binding affinity of PNA. It has been estimated that there are 1.8×10^6 PNA receptor sites per neuraminidase-treated red blood cell and both binding and hemagglutination interactions of PNA and N'RBC have been investigated¹¹⁰.

One of the most common methods for estimating the biological activity of a lectin is to compare the end point hemagglutination (the largest dilution of cells showing macroscopic evidence of agglutinated cells). Serial dilutions of N'RBC were carried out and the hemagglutination titre of both the labelled and unlabelled lectin were carried out in parallel. The end point titre occurred at a concentration of 0.5 $\mu\text{g}/\text{ml}$ for both the labelled and unlabelled product. Although the subjectiveness of the

hemagglutination only allows this method to provide a rough estimate of biological activity, it appears as if the radiolabelling procedure does not seriously affect the biological affinity of PNA.

The routine method of testing the biological activity of radioiodinated peanut lectin, was to test the percentage binding to one ml of a 2% suspension of neuraminidase-treated red blood cells (approximately 1.5×10^8 cells). The results of the experiments, measuring the binding of ^{125}I -PNA to N'RBC are presented in Table 16 and represent ten separate radioiodinations of PNA and twelve subsequent binding studies. Normal untreated red blood cells served as controls, for they have been shown to contain less than 3% of the number of receptors found in N'RBC¹¹⁰.

The radioiodinated peanut lectin was found to be very avid for binding to N'RBC with an average binding affinity of 66%. Although preliminary studies with anti-T antibodies, had indicated maximum percentages of binding to N'RBC of 8.2%, the peanut lectin showed binding capabilities as high as 84% in some cases. Since almost every N'RBC binding determination was carried out with a newly prepared ^{125}I -PNA and a freshly neuraminidase-treated lot of red blood cells, there was some degree of variation in the N'RBC binding. The specificity of the PNA-N'RBC interaction was assured by the relative lack of PNA binding to N'RBC (2.1%) in the presence of galactose. Control red blood cells also failed to show any significant binding, with an average value of 1.2%.

Table 16

In Vitro Binding of Radioiodinated Peanut Lectin
(Percentage Bound*)

Ligands	T-antigen Expressing		Control
	N'RBC	N'RBC + galactose	
Human erythrocytes (1.5×10^6 cells)	66 \pm 9 (50 to 84) n=12	2.1 \pm 0.7 (1.5 to 2.8) n=3	1.2 \pm 1.2 (0.2 to 3.8) n=11
	RI	RI + galactose	EL4 BW5-147
Tumour Cells (5×10^6 cells)	20 \pm 3 (16 to 25) n=7	1.9 \pm 0.9 (1.0 to 2.8) n=2	0.5 \pm 0.4 (0.2 to 1.0) n=5
	T-sorb	T-sorb + galactose	Blank Syasorb n=4
Immunoabsorbents (5 mg)	22 \pm 3 (17 to 27) n=5	3.1 \pm 1.2 (1.9 to 4.3) n=2	1.6 \pm 0.7 (0.9 to 2.4) n=3

* Values are expressed as a percentage of added radioactivity 125 I-PNA bound (following incubations and subsequent washes) in the form of mean \pm s.d., () range, and n=number of independent replicates.

The binding curve, of the affinity of PNA for N'RBC, was also determined. Serial dilutions of the N'RBC were prepared and 10 ng of ^{125}I -PNA was added to each dilution of cells. These binding studies were carried out in triplicate at each cell concentration. The binding curve obtained is illustrated in Fig. 12 and indicates that maximum binding has not yet been attained with the 1.5×10^8 cells contained in one ml of a 2% suspension of N'RBC.

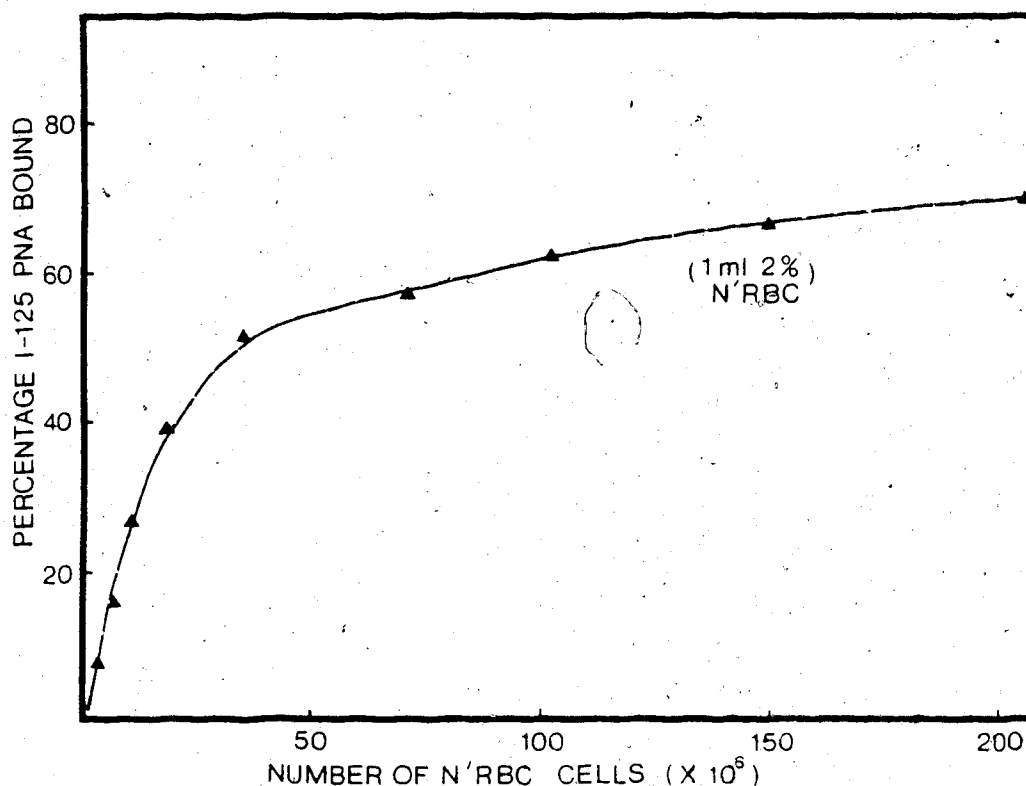


Fig. 12 Binding of ^{125}I -PNA to serial dilutions of neuraminidase-treated human erythrocytes.

The in vitro binding of ^{125}I -PNA was tested with another membrane-associated glycoprotein ligand, the RI tumour cells. As indicated in Table 16, the peanut lectin was shown to have

a very high binding affinity for RI cells, with an average 20 percent bound. In another binding determination, utilizing 1×10^7 cells rather than 5×10^6 tumour cells, $39 \pm 0.9\%$ (n=3) of the ^{125}I -PNA remained bound to the cell pellet following 4 PBS washes. The specificity of binding was indicated by a 90 percent inhibition of binding in the presence of galactose. The EL4 tumour cell line was used as a control for tumour cell binding in both in vivo and in vitro experiments. A very low binding, averaging 0.5%, was observed for EL4 cells as illustrated in Table 16. This confirmed the lack of PNA-EL4 interaction, when determined by immunofluorescence studies (Bray, unpublished data). Another control tumour cell line, BW5-147, was also found to have a very low binding affinity for PNA. Four separate radiolabelled peanut lectin preparations revealed an average percentage binding of only $0.27 \pm 0.15\%$ to the BW5-147 murine tumour cell line.

The last ligand tested for peanut lectin binding was the synthetic T immunoadsorbent, and as evidenced in Table 16, PNA bound to the T-sorb to a much greater extent (22%) than it did to a blank syrsorb (1.6%). Once again, galactose was able to inhibit the binding between PNA and the T antigen bearing ligand, so as to result in only 3.1% binding.

The various in vitro binding results of PNA indicated a specific and preferential binding of this lectin for cells and immunoadsorbents bearing T-like antigens.

6. Carbohydrate Binding Specificity of ^{125}I -PNA

Traditional methods of analyzing specific reactions of lectin and antibodies usually involve a comparison of the inhibitory ability of various mono- and oligosaccharides for either hemagglutination or glycoprotein precipitation³⁷. Erythrocyte membrane glycoproteins, and many soluble glycoproteins, appear to be very complex and are capable of binding a wide range of lectins with various saccharide-binding specificities³⁷. Synthetic immunoabsorbents, prepared by covalently linking various mono-, di- and trisaccharides, via a 9-carbon linking arm to silylated, calcined, diatomaceous earth particles (ChemBioMed, U of A), would contain only the particular carbohydrate desired. By studying the binding of ^{125}I labelled peanut lectin to various synthetic immunoabsorbents, it is possible to delineate a few of the carbohydrate binding specificities of this lectin. The final percentage of ^{125}I lectin bound to each synsorb, was determined following the 45 minute incubation and the subsequent PBS washes to remove any unbound lectin. The carbohydrate immunoabsorbents which were tested, their specific activities (μM carbohydrate per gram immunoabsorbent), and the ^{125}I -PNA binding results are all summarized in Table 17. The binding data are presented in 3 forms. The percentage of added ^{125}I -PNA which was bound to the carbohydrate-synsorbs was determined in triplicate and corrected for non-specific binding by subtracting the binding which occurred with a blank

Table 17
 Carbohydrate Binding Specificity
 of 125 I-PNA Using Synthetic Immunoadsorbents

Carbohydrate Specificity and $\mu\text{M}/\text{gram}$ of Immunoadsorbent	Percentage* Bound per 20 mg Synsorb	Percentage Bound per 0.1 μM Carbohydrate	Binding** Relative to Galactose
β -Gal (4.52 $\mu\text{M}/\text{g}$)	8.623 \pm 0.657	0.191	1.00
β -Glc (0.28 $\mu\text{M}/\text{g}$)	0.326 \pm 0.112	0.116	0.61
α -GalNAC(1+3) β -Gal (0.3 $\mu\text{M}/\text{g}$)	< 0	< 0	< 0
β -Gal(1+3) β -GlcNAC (0.48 $\mu\text{M}/\text{g}$)	0.676 \pm 0.105	0.141	0.74
β -Gal(1+3) β -GalNAC (0.58 $\mu\text{M}/\text{g}$)	42.61 \pm 1.51	7.347	38.47
β -Gal(1+3) α -GalNAC (0.27 $\mu\text{M}/\text{g}$)	25.50 \pm 2.88	9.444	49.44
β -GlcNAC(1+3) β -Gal(1+4) β -Glc (0.35 μM)	2.205 \pm 0.301	0.630	3.30

* Corrected for cpm bound to blank immunoadsorbent mean \pm one s.d. of 3 individual determinations.

** Relative binding ability standardizing to a galactose-binding ability of 1.0.

immunoabsorbent (no attached sugar residues). Since all of the synsorb exhibited various degrees of sugar incorporation, all of the binding data was standardized for specificity inter-comparisons, by calculating the percentage bound per 0.1 μM carbohydrate. Finally, galactose was assigned a binding ability of 1.0 and the binding abilities of all other sugars were calculated relative to galactose on a per μM basis.

The binding of ^{125}I -PNA to the immunoabsorbents, when compared to the galactose-synsorb, was found to be similar to relative inhibition potencies towards precipitation or hemagglutination, found by other investigators^{59, 60, 109, 258} and presented in Table 4 of the literature survey section. The immunodeterminant disaccharide of the T antigen was reported by previous workers to have an inhibitory potency, relative to galactose, of 50⁵⁹ and 54.5⁶⁰. In this study, the binding ability to the synthetic T-immunoabsorbent, was found to be 49.4 relative to galactose. This relative binding occurred when the disaccharide $\beta\text{-D-Gal}(1\rightarrow3)\text{-GalNAc}$ was covalently linked in the α position to the bridging arm of the synsorb. A lower binding ability of 38.5 was found when the disaccharide was covalently bound by a β linkage to the bridging arm. This agrees with the findings of Kaifu and Osawa²⁵⁸ when they studied the inhibition of PNA binding to N'RBC. A better inhibitory ability was found for the synthetic carbohydrate chains when the T determinant was attached via an α , rather than β , linkage to the 3-0-

position of N-tosyl-L-serine²⁵⁸.

Of the other carbohydrates tested, none showed a binding ability exceeding 10% of that found between PNA and the T determinant. For weakly bound carbohydrates, where nonspecific interactions may contribute significantly to binding, wider fluctuations in the affinity are often found between one specificity study and another. While Pereira *et al.*⁵⁹ determined a relative inhibition of <1.5 for α -GalNAc(1 \rightarrow 3)- β -Gal, the peanut lectin was found to bind less to this specific immunoadsorbent than to a blank synsorb, so that the corrected percentage bound was actually found to be less than zero, in this study. The α anomeric configuration of the terminal galactose moiety and the blocked hydrogen-binding C-2 substituent may both have contributed to the very low binding of PNA to this disaccharide.

The importance of the configuration of the C-4 hydroxyl in the penultimate pyranose is evident when comparing the relative binding of PNA to β -Gal(1 \rightarrow 3)D-GlcNAc and β -Gal(1 \rightarrow 3)D-GalNAc. By substituting glucose for galactose as the penultimate sugar, the binding decreased from 49.4 to 0.74. Pereira *et al.*⁵⁹ found a similar decrease of 54.5 to 0.6 in their inhibition studies. The last compound listed in Table 17, β -GlcNAc(1 \rightarrow 3) β -Gal(1 \rightarrow 4) β -Glc, was not tested by other investigators^{59, 60, 109} although a relative potency of 3.30 determined in this study is close to the value of <3.1 found for α -GlcNAc(1 \rightarrow 4) β -Gal(1 \rightarrow 3)GlcNAc⁶⁰.

The study of binding specificity of a lectin by simply measuring the percentage binding to synthetic immuno-adsorbents appears to offer an efficient rapid means of analysis that produces results similar to those seen with the accepted inhibition studies.

A very specific interaction of PNA with the T-immuno-determinant disaccharide was evident in this study and supports the in vitro tumour and N'RBC cell binding studies. Therefore, further studies were warranted to test the in vivo localizing ability of radioiodinated peanut lectin in tumours expressing T-like antigens on their cell surfaces. Therefore, CBA/CAJ mice with RI tumours were utilized to test the potential of radioiodinated peanut lectin for the radioimmunodetection of cancer.

7. Biodistribution of ^{125}I -PNA in CBA/CAJ Mice Bearing RI Tumours

The peanut lectin, used in this biodistribution study, was radioiodinated with an efficiency of 60% to provide a specific activity of 185 kBq/ μg (≈ 0.26 atoms iodine/molecule). In vitro analysis of this particular preparation of ^{125}I -PNA revealed that $66.6 \pm 1.2\%$ bound to a one ml 2% suspension of N'RBC and 39% of the radioactivity was bound to 1×10^7 RI tumour cells. Retention of biological activity following radioiodination was demonstrated by a preserved hemagglutination titre to N'RBC. Over 98 per cent of the radioactivity in the injected product could be precipitated by trichloroacetic acid. The CBA/CAJ male mice

had received a subpannicular inoculation of 5×10^5 RI cells, in the right flank. This resulted in well defined solid tumours, averaging 250 mg, at 11 to 15 days post-inoculation. Lugol's solution was added to the animals' drinking water, commencing two days prior to i.v. injection. The tumour-bearing mice received a 0.2 ml caudal i.v. injection of 50 kBq ^{125}I -PNA. At 3, 8, 24 and 48 hours following injection, the mice were sacrificed by cardiac puncture/exsanguination under light ether anaesthesia. The liver, spleen, kidneys, pancreas and tumour were dissected as entire organs. The stomach, duodenum (first 2 cm of GIT) and remaining GIT were assayed with their contents. The blood sample content of radioactivity was extrapolated to the entire blood pool on the basis of animal body weight (entire blood pool = 6.5% body weight)²⁵⁸, and the skeletal muscle sample was taken from the hind thigh contralateral to the tumour site. The tissue samples, along with injection standards, were assayed for radioactivity in an automated gamma spectrometer (Beckman 8000 gamma counter) utilizing the coincidence method for absolute determination of ^{125}I radioactivity.

The results of the biodistribution study are presented as a percentage of injected ^{125}I -PNA per gram wet tissue and on the basis of entire organ accumulation in Tables 18 and 19, respectively. The values are given as the mean and standard deviation of the 6 to 8 mice sacrificed per time period.

TISSUE DISTRIBUTION OF I-125 PNA IN CBA/CAJ MICE BEARING RI TUMOURS

TUMOUR	(PERCENT DOSE PER GRAM TISSUE)				(PERCENT DOSE PER INTACT ORGAN)					
	TABLE 18	3HR N=7	8HR N=6	24HR N=8	48HR N=8	TABLE 19	3HR N=7	8HR N=6	24HR N=8	48HR N=8
	%DOSE/GRAM STAN.DEV.	3.894 1.073	2.524 1.151	0.618 0.283	0.178 0.064	%DOS/ORG STAN.DEV.	0.572 0.285	0.557 0.316	0.095 0.051	0.077 0.056
BLOOD	%DOSE/GRAM STAN.DEV.	1.543 0.453	0.926 0.590	0.124 0.058	0.011 0.003	%DOS/ORG STAN.DEV.	2.759 0.814	1.621 1.028	0.220 0.114	0.020 0.006
LIVER	%DOSE/GRAM STAN.DEV.	0.748 0.155	0.441 0.231	0.140 0.021	0.064 0.009	%DOS/ORG STAN.DEV.	0.976 0.173	0.611 0.289	0.188 0.034	0.101 0.011
SPLEEN	%DOSE/GRAM STAN.DEV.	4.799 0.569	2.706 0.439	0.765 0.118	0.191 0.042	%DOS/ORG STAN.DEV.	0.649 0.110	0.403 0.089	0.105 0.014	0.028 0.005
KIDNEY	%DOSE/GRAM STAN.DEV.	8.931 3.757	3.849 1.453	0.917 0.145	0.323 0.079	%DOS/ORG STAN.DEV.	3.543 1.347	1.570 0.493	0.343 0.060	0.125 0.029
STOMACH	%DOSE/GRAM STAN.DEV.	8.889 1.457	8.568 6.199	1.030 0.611	0.075 0.027	%DOS/ORG STAN.DEV.	2.724 0.742	2.928 1.471	0.343 0.199	0.039 0.011
PANCREAS	%DOSE/GRAM STAN.DEV.	0.977 0.192	0.562 0.365	0.058 0.024	0.009 0.002	%DOS/ORG STAN.DEV.	0.062 0.038	0.074 0.063	0.011 0.005	0.001 0.000
DUODENUM	%DOSE/GRAM STAN.DEV.	2.495 0.785	1.943 1.432	0.146 0.059	0.018 0.005	%DOS/ORG STAN.DEV.	0.299 0.085	0.234 0.183	0.018 0.009	0.002 0.001
GIT	%DOSE/GRAM STAN.DEV.	1.431 0.332	0.854 0.490	0.082 0.025	0.012 0.003	%DOS/ORG STAN.DEV.	3.717 0.791	2.156 1.079	0.193 0.059	0.033 0.006
MUSCLE	%DOSE/GRAM STAN.DEV.	0.339 0.086	0.145 0.110	0.024 0.013	0.005 0.002					

The biodistribution data reveal that most tissues exhibited a relatively rapid elimination of ^{125}I -PNA, although the tumour retention was greater than most other tissues. One gram of blood contained only 0.01% of the injected activity at 48 hours and this was less than one per cent of the radioactivity initially present at 3 hours. The tumour, on the other hand, retained at 48 hours, almost 5 per cent of the radioactivity initially present at 3 hours, so that one gram of tumour tissue contained 0.62% and 0.18% of the injected ^{125}I -PNA at 24 and 48 hours, respectively. On a gram basis, the only tissues which exhibited a consistently greater uptake of ^{125}I -radioactivity than the tumour, were the kidney and spleen. An initially high stomach uptake of ^{125}I , decreased rapidly with time, so that only 0.8% of the 3 hour stomach radioactivity was retained at 48 hours. The liver, pancreas and muscle were found to have a relatively low uptake of ^{125}I -PNA.

The peanut lectin demonstrated a very rapid whole body elimination. The percentage of injected radioactivity remaining in the mouse, at time of sacrifice, was only $42 \pm 13\%$, $29 \pm 15\%$, $4.4 \pm 1.6\%$ and $1.3 \pm 0.4\%$ at 3, 8, 24 and 48 hours, respectively. The blood clearance was also very rapid, as indicated by the biodistribution data. At 8 hours only $5.4 \pm 0.7\%$ of the radioactivity remaining in the body was contained in the entire blood pool and by 48 hours this had fallen to $1.8 \pm 0.4\%$.

The rapid blood clearance, when combined with a tumour retention greater than that of most other organs, resulted in very favorable tumour-to-background ratios. The time course of both tumour:muscle and tumour:blood ratios of radioactivity are shown in Fig. 13. Additional tissue:blood and tissue:muscle ratios are presented in Appendix II.

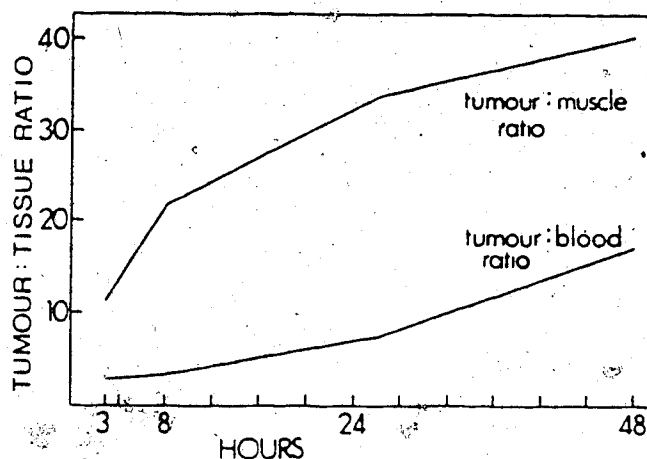
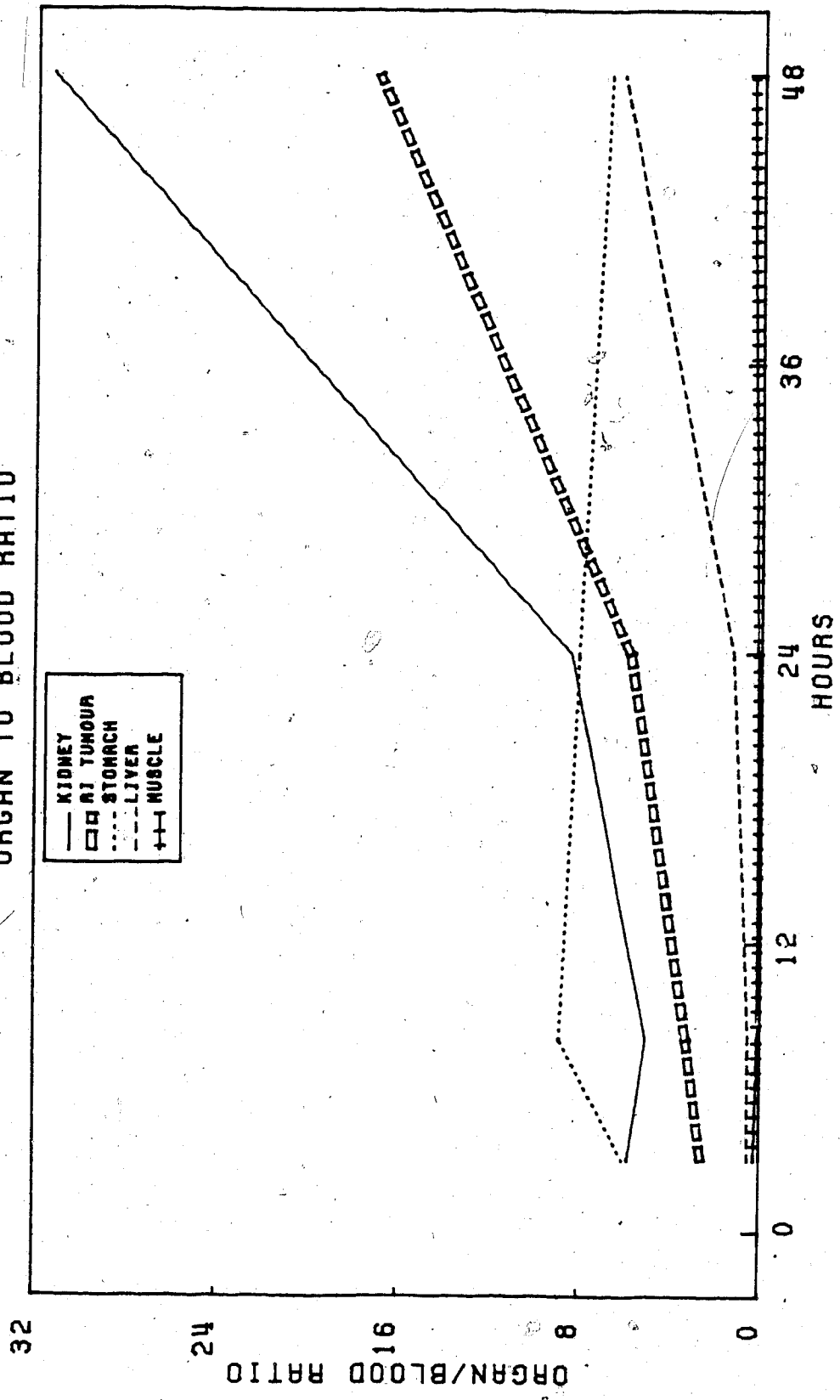


Fig. 13 Tumour:Muscle and Tumour:Blood Ratios of Radioactivity following ^{125}I -PNA Injection into Mice bearing RI Lymphoma Tumours

Average tumour:blood ratios of radioactivity increased steadily as a function of time. A much more significant retention of radioactivity in the tumour than in the blood resulted in an average tumour:blood ratio of 17:1 at 48 hours. The tissue:blood ratios of ^{125}I -PNA are compared for the tumour and various other organs as a function of time in

FIG. 14

BIODISTRIBUTION OF I-125 PEANUT LECTIN
ORGAN TO BLOOD RATIO

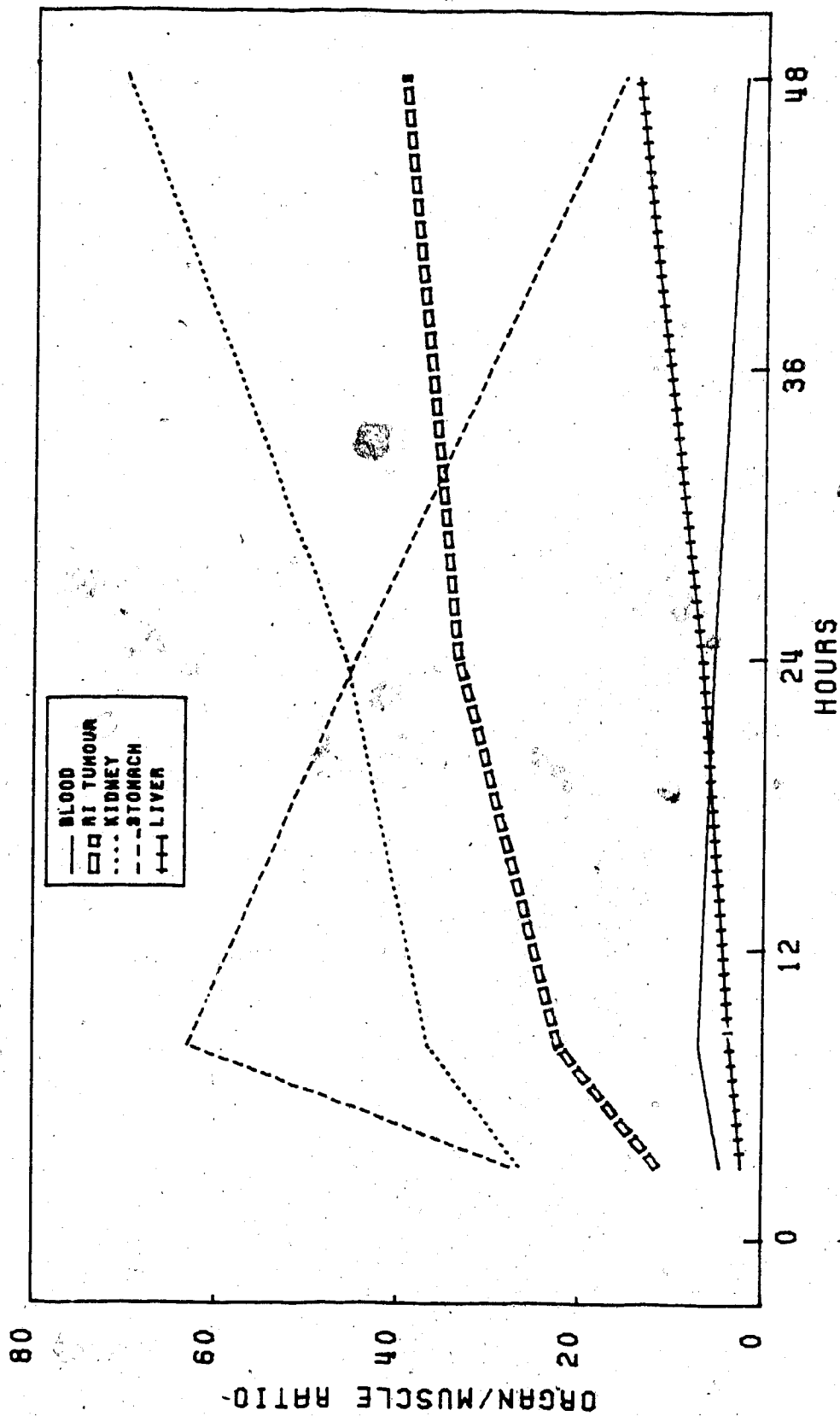


RI lymphoma bearing CBA/CAJ mice in Fig. 14. As is evident from the graph, a steadily increasing tumour: blood ratio occurred as a function of time. Metabolism of the radioiodinated peanut lectin resulted in production of free iodide and the in vivo biological breakdown of ^{125}I -PNA was found to be very rapid. Trichloroacetic acid precipitation of the plasma samples revealed the presence of 70% free iodide in the plasma at 3 hours and 80% after 8 hours. This was reflected in the organ distribution, in that the stomach had maximum relative uptake at 8 hours. The important role that the kidney plays in the elimination of free iodide probably leads to the high kidney: blood ratio, also illustrated in Fig. 14. Muscle: blood ratios were very low throughout the study period.

Another background tissue, used for comparison of tissue radioactivity, was the skeletal muscle, and as evidenced in Fig. 13, the tumour: muscle ratio also increased steadily as time progressed. Average tumour: muscle ratios that were already 13:1 at 3 hours, reached an average value of 40:1 at 48 hours with individual ratios as high as 65:1. The tissue: muscle ratio of various tissues is illustrated in Fig. 15. The contribution of metabolized ^{125}I -PNA to tissue radioactivity is once again evident in the stomach and kidneys. The liver: muscle ratio is generally only 30% of the radioactivity ratio shown by tumour: muscle.

Since the radioactivity was determined for the remaining carcass and for the tissues-of-interest, it was

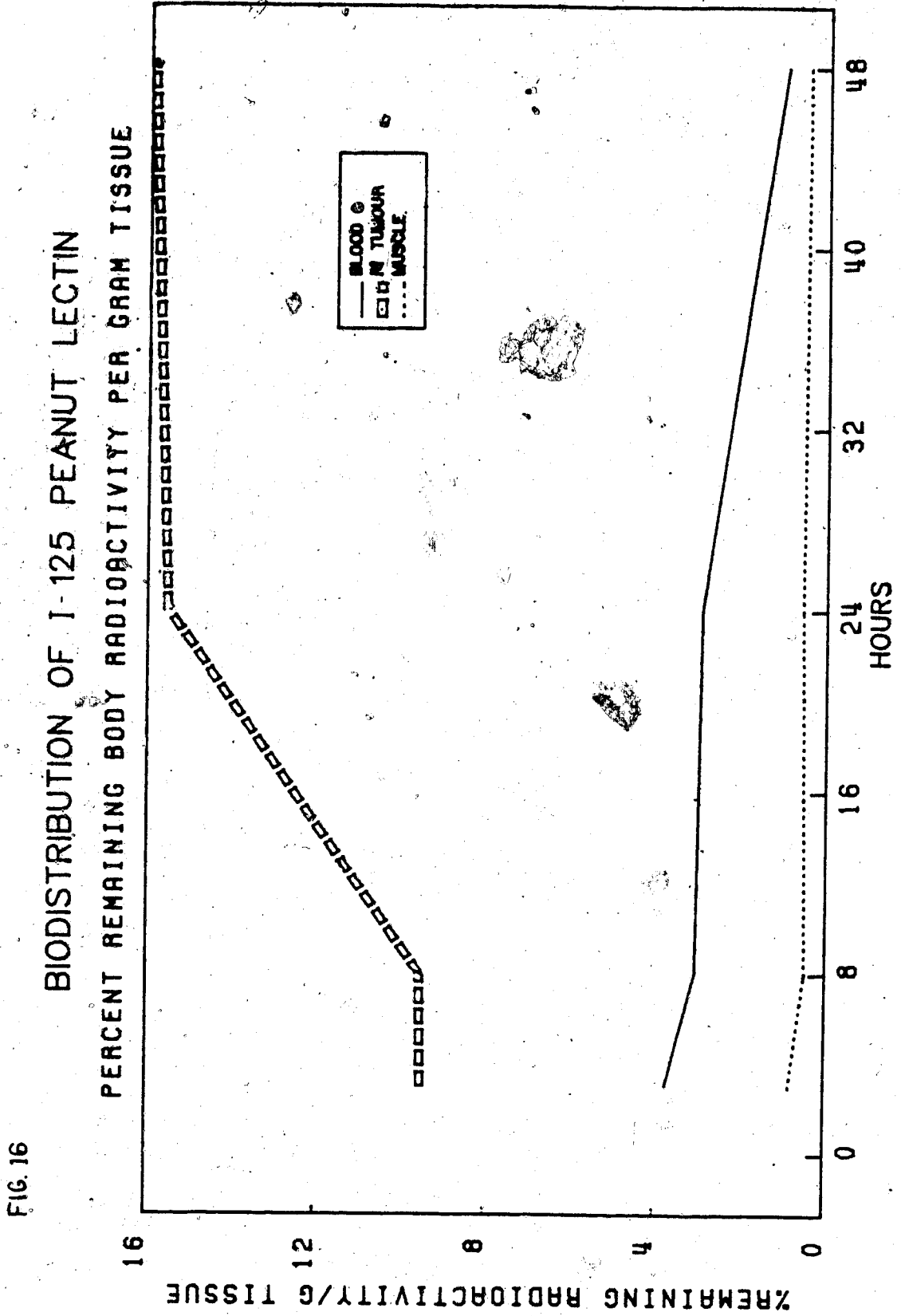
FIG. 15 ORGAN TO MUSCLE RATIOS FOR I-125 PEANUT LECTIN



possible to calculate the percentage of radioactivity in various tissues, based on the radioactivity remaining in the body at the time of sacrifice. The percentage of remaining dose per gram of tissue, is illustrated in Fig. 16 for the tumour, blood and muscle. At 3 and 8 hours, the tumour contained about 10% of the remaining body radioactivity, per gram of tumour tissue. By 24 hours, the overall elimination of radioactivity from the whole body was greater than that from the tumour. Such selective tumour retention resulted in 16% of the remaining body radioactivity being contained per gram of tumour tissue at 24 and 48 hours post- ^{125}I -PNA-injection. The very low muscle retention of ^{125}I -PNA and a blood clearance of radioactivity which was faster than whole body elimination is also illustrated in Fig. 16.

The amount of radioactivity contained in various entire organs at 24 hours, expressed as a percentage of the ^{125}I -PNA remaining in the body, was as follows: tumour ($2.6 \pm 1.6\%$), liver ($4.7 \pm 1.4\%$), spleen ($2.6 \pm 0.7\%$), both kidneys ($8.5 \pm 2.5\%$), stomach ($8.0 \pm 3.8\%$), pancreas ($0.26 \pm 0.12\%$), small and large intestine ($4.7 \pm 1.4\%$). Preliminary biodistribution studies had revealed that the thymus, brain, fat and bone showed very low uptake of radioiodinated peanut lectin.

The serial gamma camera images reflected the tissue biodistribution data. Posterior views of 20,000 counts, collected with a Pho Gamma IV camera (pinhole collimator) at 6, 24, 48 and 72 hours are shown in Plate I. A diffuse whole body distribution was seen on the initial day with



some localization evident in the tumour. The stomach, left kidney and spleen, which all lie in close proximity and in fact overlie one another in the posterior view, combine to produce a "hot spot" on the left side of the animal. Rapid clearance from the blood, stomach and muscle and a relatively slower clearance in the tumour, allowed an even clearer delineation of the tumour by 24 and 48 hours. The tumour shows the most intense localization in the body at 48 and 72 hours. No blood pool subtraction was necessary for successful imaging.

Dissection of an animal imaged at 72 hours (Plate I) revealed that 37% of the total radioactivity remaining in the body was contained within the tumour. The tumour:muscle ratio in this particular mouse was 45:1 and the tumour:blood ratio was 7.5:1. The "hot spot" in the region of the neck corresponds to the salivary glands, which contained 5.6% of the total activity remaining in the body. Levels of radioactivity in other organs at this time were: thyroid 1%; stomach 2.7%; kidneys 3.5%; lungs 1.6% and testes plus seminal vesicles 1.2%.

Although localization of the radioactivity in the kidney, stomach and salivary gland was probably due to the radioiodide from the metabolized ^{125}I -PNA, there may also be some ^{125}I -PNA specific binding in these tissues. The peanut lectin has been used in vitro for the histochemical staining of mouse organ sections and PNA reactive glycoproteins have been found in the stomach, salivary

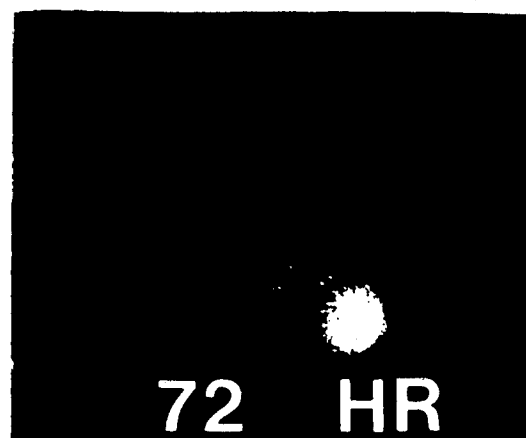
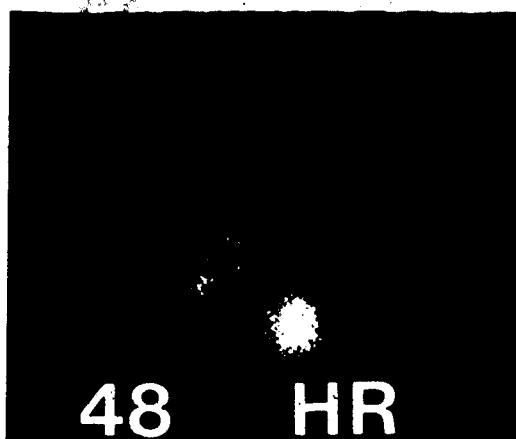
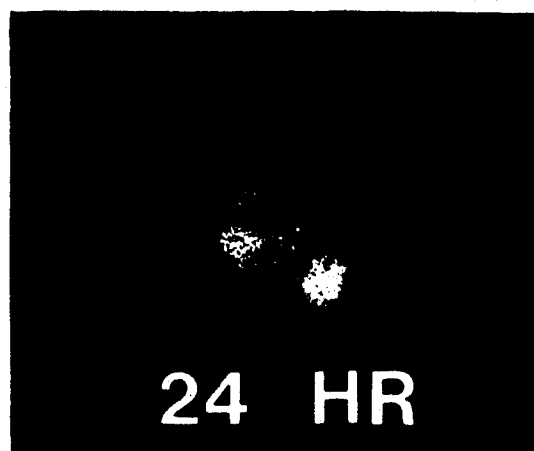


Plate I. Serial Gamma Camera Images of an RI Lymphoma Tumour-Bearing Mouse Injected i.v. with 350 kBq ^{125}I -PNA. (Pinhole collimator; 20,000 counts per image).

glands, GIT, kidney, lung, pancreas and sex organs^{166, 167, 259, 260}. However, as mentioned in the literature survey section, results of in vitro binding to tissue sections are not easily extrapolated to in vivo localization studies. Tissue sections reveal sites not normally available to a macromolecule. The intracellular cytoplasm and Golgi zones are readily available in tissue slices, for interaction with a protein molecule. The final sialic acid-capping reaction, in the sequential synthesis of normal cell surface glycoproteins, occurs within the Golgi apparatus¹⁴⁴. The T antigen is believed to be a precursor of normal cellular glycoproteins¹⁷⁵, and as such would be expected to be found, in the unmasked form, within the secretory bodies and Golgi apparatus of normal cells. These sites are not normally available for macromolecular interaction in intact cells, but are exposed in tissue sections. Indeed, one of the major sites of PNA interaction in histochemical studies of mouse tissues was found to be the Golgi zones and stored secretory bodies^{166, 167, 259, 260}.

Peanut lectin reactive sites have also been found, in in vitro studies, at extracellular locations. The mucous secretions and luminal surface of epithelial linings in the GIT, pancreatic and genitourinary tracts have been found to bind PNA when mouse tissue sections were examined in vitro^{166, 167, 259}. When tissue sections of human breast tissue were examined for in vitro binding of PNA, they also demonstrated a PNA-binding pattern which tended to be

confined to luminal surfaces of epithelial cells and their secretions^{36, 37, 38, 47}. The finding of PNA reactive sites in normal, as well as malignant tissue, has resulted in some investigators questioning the designation of the T antigen, and the PNA receptor, as a tumour-associated antigen³⁸.

Although these sites are readily available for peanut lectin interaction when tissue sections are tested in vitro, the luminal surfaces are essentially "outside" the body and are usually immunologically privileged and poorly vascularized. The poor contact with blood and lymph in these locations may not allow the in vivo localization and binding of a macromolecule, such as radioiodinated peanut lectin, before the protein is metabolized.

The importance of the vascularity, macromolecular accessibility of a tissue is demonstrated in the present investigation, by the failure of in vivo localization of radioiodinated PNA in the thymus of CBA/CAJ mice. Although 65 to 90 per cent of mouse thymocytes have been found to bind PNA when tested histologically or in cell suspensions^{115-117, 260} only 0.003% of the injected dose of ¹²⁵I-PNA was found in the thymus, 24 hours post-injection. The thymus possesses a specialized vasculature, with an especially thick basement membrane and epithelial cell layer^{99, 261}. The epithelial membrane acts as a barrier to impede passage of macromolecules, such as the peanut lectin, to the PNA reactive cells in the parenchymal interior.

The vascularity of the tumour also plays an important role in the accessibility of tumour cell surface antigens for interaction with radiolabelled lectins and other protein macromolecules. Animal tumour models, with solid subcutaneous tumours, provide tumour antigens which are located outside the host's normal vascular bed and do not allow for extremely rapid antigen-lectin interaction^{235, 241}. When this is borne in mind, the clear tumour images obtained at 24 and 48 hours and tissue biodistribution data indicate a localization and retention of the radioiodinated peanut lectin which is possibly only limited by the tumour vascularity.

The selective tumour retention and fast blood clearance of radioiodinated PNA, combine to produce tumour: blood ratios such as 17:1 and these results appear very promising when compared to other proteins and antibodies used for the radioimmunodetection of cancer. The localization of tumours using affinity purified anti-CEA (carcinoembryonic antigen) has been complicated by low tumour: blood ratios of, for example, 1.3:1 at 24 hours²⁸ and 2.4:1 at 48 hours¹⁸. The absolute requirement of computerized blood pool subtraction for successful imaging with anti-CEA has led some workers to express doubts for its routine clinical usefulness²⁷. A high blood background may also complicate studies utilizing human anti-T immunoglobulins. These antibodies are primarily of the IgM class and therefore are largely confined to the bloodstream²⁶².

The solid subpannicular RI tumours, used in studying the biodistribution of ^{125}I -PNA, were analyzed for presence of the T antigen following in vivo growth and they were found to lose the antigen to some extent. When grown in vitro in tissue culture media as a cell suspension, to 90 percent of the RI tumour cells were found to bind peanut lectin, as well as a human and rabbit preparation of anti-T (as determined by direct and indirect immunofluorescent studies of Dr. J. Bray). The binding of anti-T antibodies to RI tumours cells was detected by indirect immunofluorescence and is illustrated in Plate II.

Following 11 to 17 days growth in vivo as a solid subpannicular tumour, only between 14 and 18% of the cells expressed the T antigen, independent of the tumour mass. Therefore the expression of the PNA binding receptors on the tumour cells appears to be sensitive to the environment in which the cells grow.

Changes in cell surface glycoproteins have been documented for other tumour cell lines^{263, 265}. The TA3-Ha ascites mammary adenocarcinoma cells have been shown to shed T- and MN antigens from the cell surfaces and during transfer of the ascites form, to either suspension culture or a solid form of in vivo tumour, the tumour cells were found to lose cell surface glycoproteins^{264, 265}. The glycoprotein content of the 13762 rat mammary adenocarcinoma also appears to depend on the environment or on the condition of growth²⁶³. The major sialoglycoprotein, ASPG-1, of the

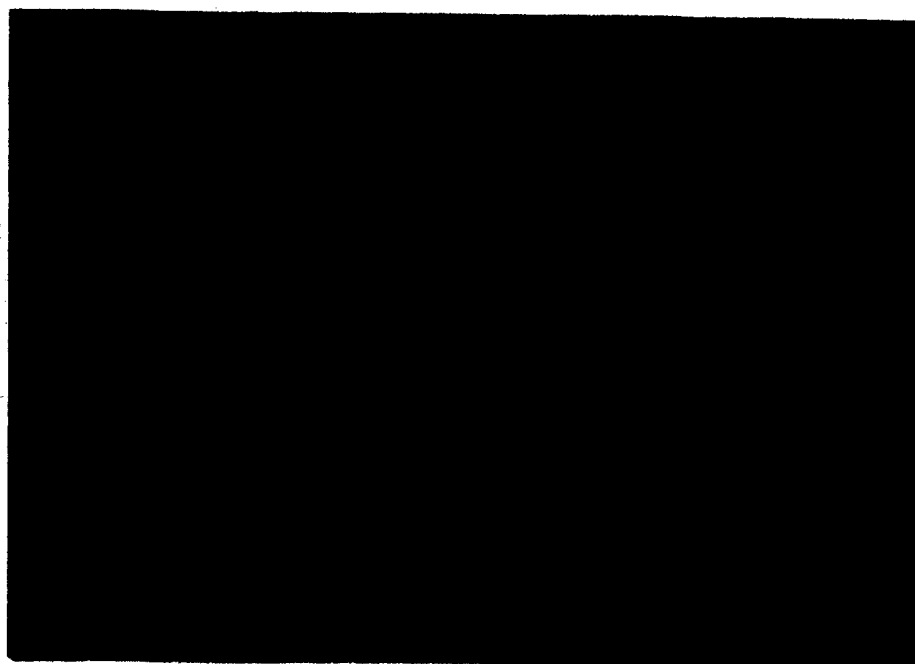


Plate II. Binding of fluorescein-labelled rabbit anti-T antibodies to RI tumour cells (courtesy of Dr. J. Bray, unpublished results.)

ascites form of 13762 rat tumour cells has been shown to contain PNA receptors²⁶³. The solid 13762 tumour, from which all the ascites sublines were derived, does not contain ASPG-1, as detected by PNA. When this tumour cell line is grown in the ascites form, the ASPG-1 glycoprotein appears to be shed from the cell surface and soluble ASPG-1 glycoprotein is found in the ascites fluid and plasma of ascites-tumour-bearing rats²⁶³. Cooper et al.²⁶⁵ has postulated that changes in cell surface glycoproteins may be related to the metastatic potential of the tumour cells.

The decreased expression of PNA receptors on RI cells, when grown as a solid subpannicular tumour rather than a cell suspension, may be a result of clonal selection from a heterogeneous population of tumour cells²⁶³. Perhaps the 10 to 30% RI cells, which do not bind ¹²⁵I-PNA when grown as a cell suspension, are the cells best adapted to grow in the environmental conditions of a solid subpannicular tumour. Another possibility is that the expression of the PNA receptor glycoprotein is altered following the change in tumour cell environment. Continued sequential passage of the RI cells as a solid subpannicular (s.p.) tumour may possibly reverse the loss of PNA-receptor expression. It was found that further s.p. transplantation of the TA3-Ha cell line as a solid tumour, led eventually to increases of sialoglycoprotein to the same level observed under ascitic conditions^{264, 265}. Further studies of the characteristic behavior of the PNA receptor on the cell surface of

RI tumour cells, in different environments will need to be carried out.

Considering the localization of ^{125}I -PNA was relatively high in an animal tumour model in which only 14 to 18% of the cells of the tumour cell mass express PNA receptors, radiolabelled peanut lectin appears to present great potential as an agent for the radioimmunodetection of cancer.

8. The In Vivo Specificity of Tumour Localization of ^{125}I -PNA

In order to gain a better insight into the mechanism of in vivo PNA localization in RI tumours, it was desirable to determine the relative contribution of specific and non-specific uptake in animal tumour models. The specificity of in vitro binding to RI tumour cells was demonstrated by the fact that addition of galactose produced an inhibition of ^{125}I -PNA binding to RI tumour cells in culture and by the absence of binding when tumour cells, EL4, not expressing the T antigen were tested. The paired-label technique of Pressman²⁵⁰ was utilized to distinguish specific and non-specific protein localization in tumours and other tissues.

Although antibodies within a certain class, eg. IgG, of different binding specificities, are structurally similar^{33, 99}, a non-specific lectin is not readily available to act as a control for studies of peanut lectin binding. Therefore, a protein molecule of similar molecular weight, F(ab')₂ non-specific rabbit IgG fragment, was chosen. This fragment

has a molecular weight of 100,000 to 105,000 and is prepared from IgG molecules, by cleavage of the Fc portion of IgG, with pepsin treatment²⁶⁶. The Fc fragment is involved in the binding of complement, tissues and macrophages^{99, 267}, so that its removal in the preparation of F(ab')₂ fragments will hopefully decrease nonspecific accumulation in both normal tissues (lack of Fc binding) and tumours (lower molecular weight).

The F(ab')₂ nonspecific rabbit IgG fragment therefore was chosen as a control protein to be labelled with I-131 and then injected simultaneously with ¹²⁵I-PNA. Differential radioactive counting of tissue samples for the two different iodine radioisotopes then allows for analysis of the specificity of PNA localization.

Two animal tumour models were used to analyze the specificity of tumour localization. The EL4 mouse lymphoid tumour cell line does not express receptors for the peanut lectin and when tested in vitro, radioiodinated peanut lectin had very low affinity for this tumour cell line (Table 16). Therefore, 6×10^5 viable EL4 cells were injected s.p. in C57 black mice, to provide a solid subpannicular tumour 10-12 days later. The C57/Black mice, bearing EL4 tumours, then served as a control animal-tumour model for the CBA/CAJ mice, bearing RI cells.

In separate biodistribution studies, both sets of tumour-bearing animals were injected simultaneously with 3 MBq ¹²⁵I-PNA (165 KBq/ μ g) and 3.5 MBq ¹³¹I-F(ab')₂. Between

4 and 6 animals in both groups of tumour bearing mice were dissected at the time periods of 24 and 48 hours.

Additional CBA/CAJ mice were sacrificed at 8 and 72 hours.

The tissue samples and injection standards were analyzed for I-131 and I-125 radioactivity, as described earlier.

The specificity of the protein localization in the two different tumour animal models was analyzed by calculating the specificity indices. The ratio of relative tissue: blood radioactivity concentration obtained for radio-labelled peanut lectin, was divided by the corresponding tissue: blood ratio for the control protein, as outlined in the following equation:

$$\text{specificity index} = \frac{\text{tissue: blood } ({}^{125}\text{I-PNA})}{\text{tissue: blood } ({}^{131}\text{I-F(ab')}_2)}$$

Figures 17 and 18 illustrate the specificity indexes found for both tumour animal models at 24 hours, while figures 19 and 20 illustrate those observed at 48 hours. The numerical specificity indices, of figures 17 to 20, are listed in Appendix III. The percentage injected dose per gram of tissue (utilized in calculating these ratios) and the percentage injected dose incorporated per entire organ are outlined in Appendix IV and V, respectively.

The specificity index was compared for the CBA/CAJ mice (with the T antigen bearing RI tumours) and C57/Black mice (with the non-T antigen expressing EL4 tumours) using an unpaired t-test. Statistically ($p < 0.01$) higher specificity indices were found in the tumour and spleen of the RI tumour bearing mice, as compared to the EL4 tumour

FIG. 17

PNA SPECIFICITY INDICES AT 24 HOURS
(IN TUMOUR, MUSCLE, THYROID, STOMACH, GIT, & LUNG)

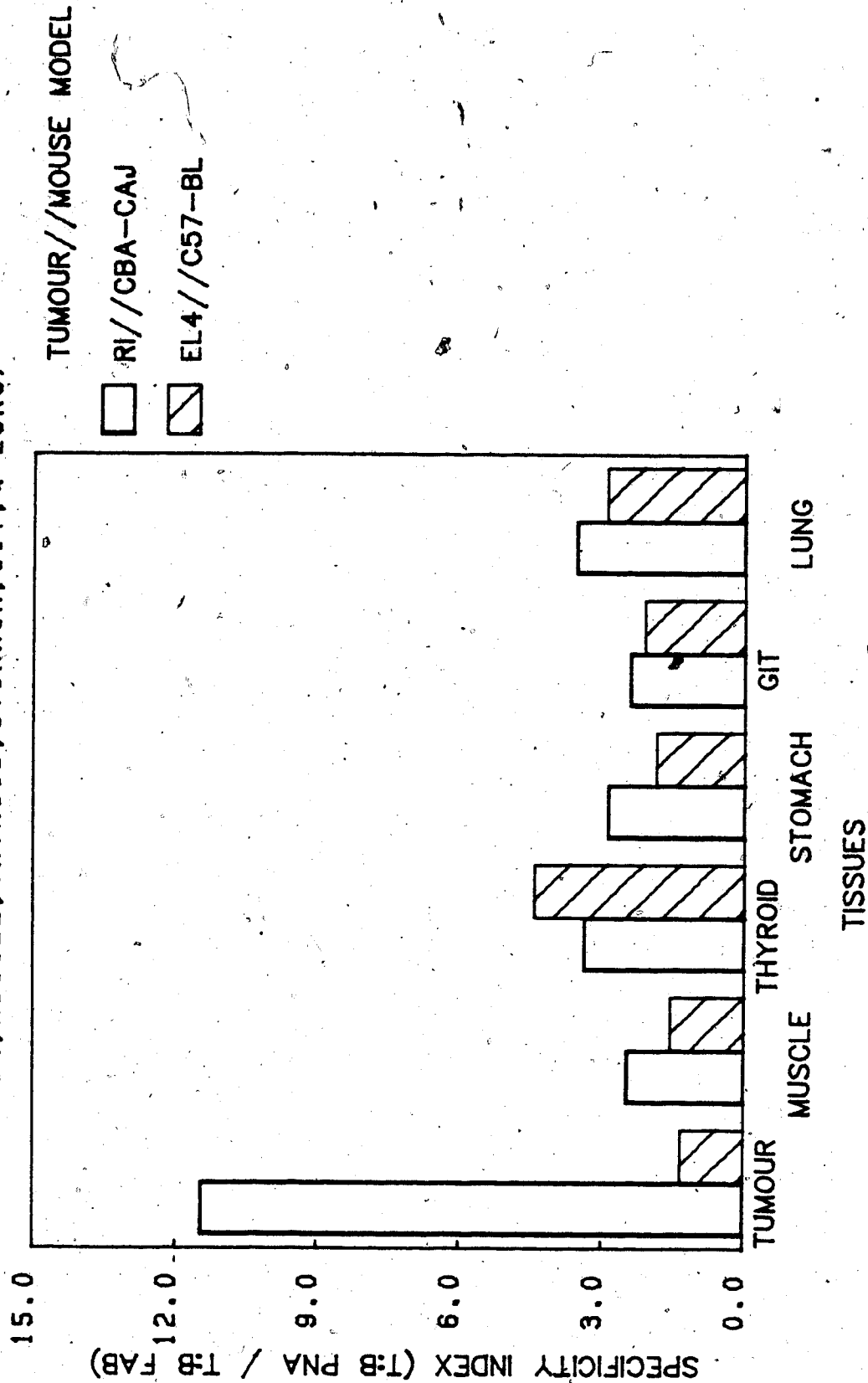


FIG. 18

PNA SPECIFICITY INDICES AT 24 HOURS
(IN SPLEEN, KIDNEY, LIVER, SALIVARY, SEX, THYMUS)

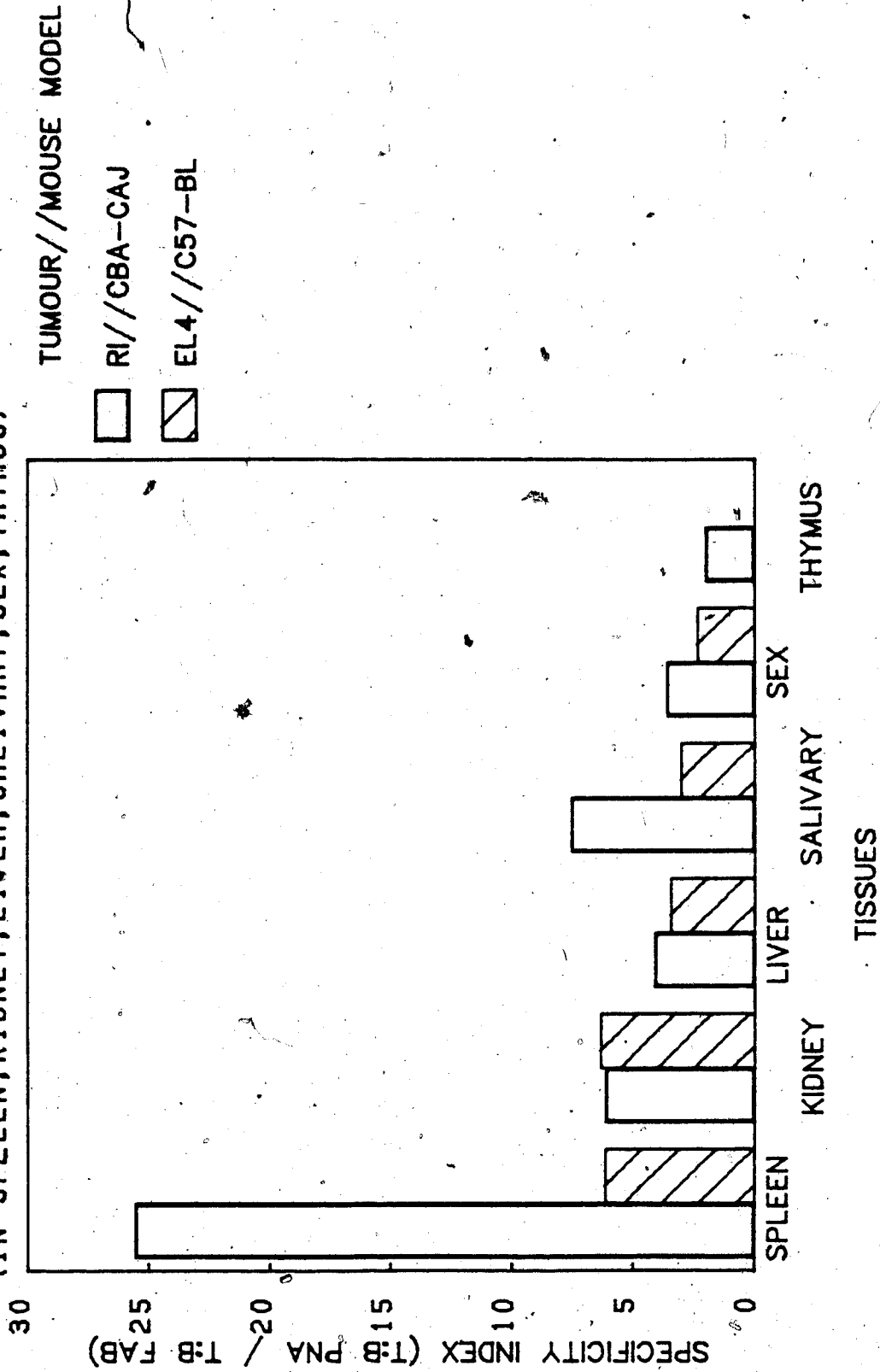


FIG. 19

PNA SPECIFICITY INDICES AT 48 HOURS
(IN TUMOUR, MUSCLE, THYROID, STOMACH, GIT & LUNG)

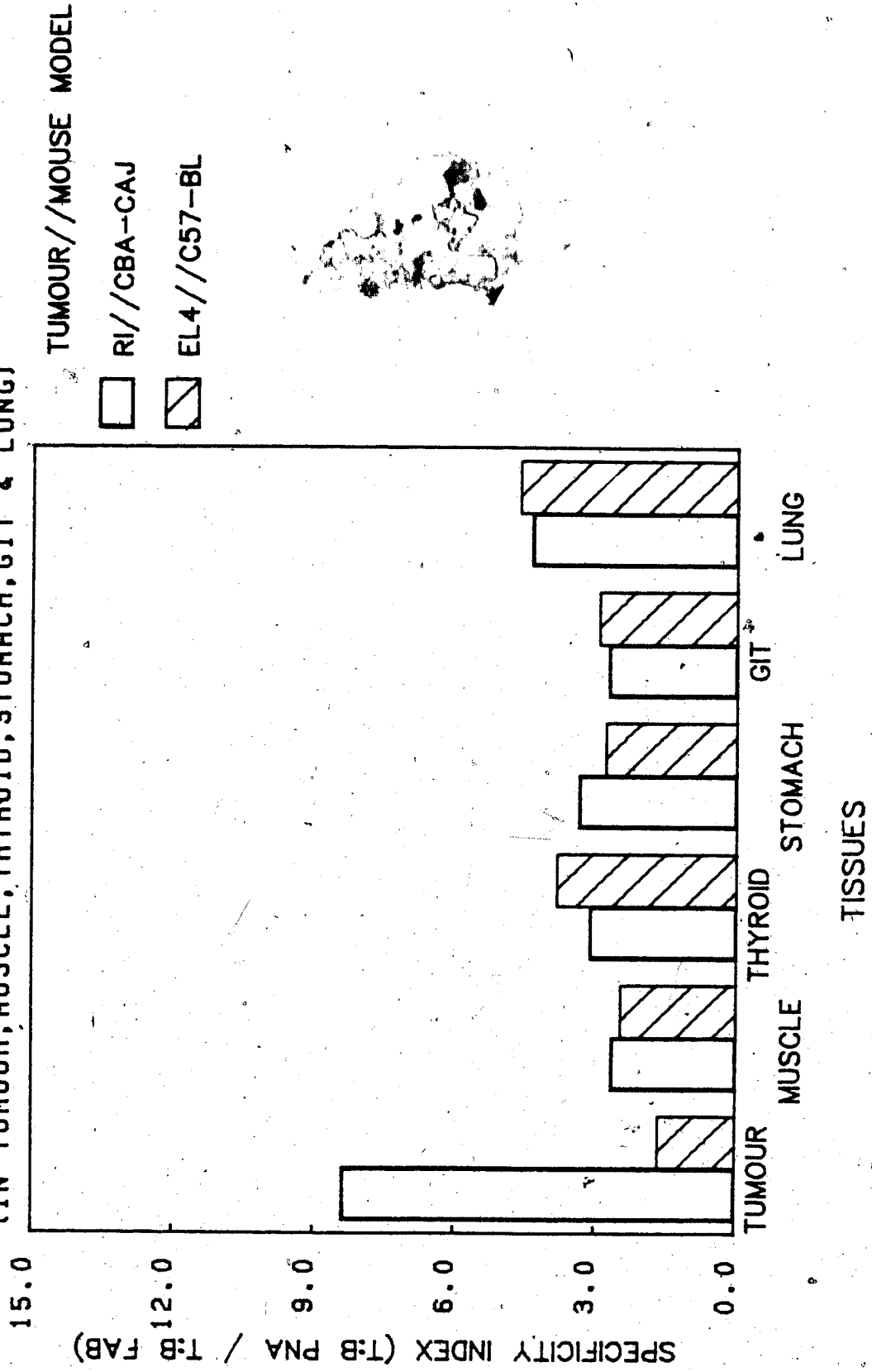
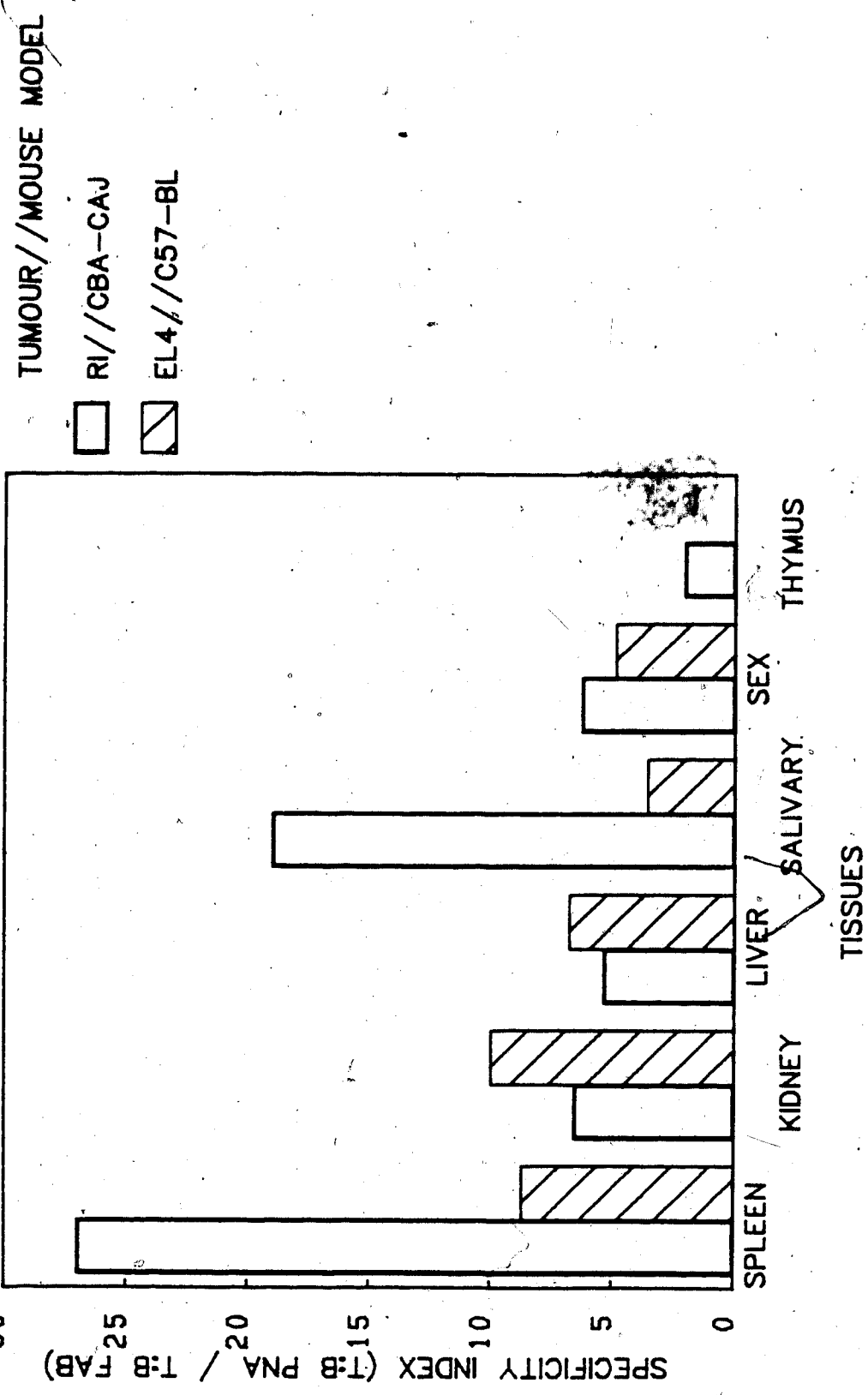


FIG. 20

PNA SPECIFICITY INDICES AT 48 HOURS
(IN SPLEEN, KIDNEY, LIVER, SALIVARY, SEX, THYMUS)



bearing mice at 24 and 48 hours and the salivary glands at 48 hours. None of the other tissue specificity indices were found to be significantly different ($p < 0.01$) between the two animal tumour models.

As seen in Fig. 17, at 24 hours the tumour exhibited a specificity index of 11.46 ± 1.14 in CBA/CAJ mice with RI tumours. Therefore, the $F(ab')_2$ fragment, which has a similar molecular weight to PNA but not the specific glycoprotein binding ability of PNA, could only account for about 8.5% of the PNA localization (expressed as a tumour to blood ratio of radioactivity) that occurred in the RI tumour. However, when an animal tumour model was used in which the tumour did not express the T-like specificities, this preferential tumour localization of PNA was no longer evident. Therefore a specificity index of only 1.34 was observed in the C57 black mice, bearing EL4 tumours, and 75% of the PNA uptake in this tumour is possibly due to non-specific localization.

These results point to a preferential localization and retention of radiolabelled peanut lectin only within tumours presenting cell surface receptors with which the PNA may specifically interact. A specificity index of 11.5 at 24 hours post-injection, appears very promising when compared with other radioimmunodetection studies in animal tumour models. Moshakis *et al.*²⁸ examined the localization of radiolabelled anti-CEA antibodies in mice bearing xenografts of human breast tumours and a specificity index

of 3.7 was achieved at 24 hours, using normal IgG as the control protein. Monoclonal antibodies prepared to human teratoma xenografts, resulted in specificity indices of localization of 2.9 at 24 hours and 5.1 at 48 hours²⁶⁸. Human colonic xenografts in mice²⁵³ and hamsters²⁶⁹ were used as animal tumour models for anti-CEA radiolocalization studies and 3 days were required before average specificity indices of 2.8 and 7.7, respectively, were achieved. The peanut lectin therefore appears to achieve a specific tumour localization at 24 hours, 3 to 4 fold higher than that obtained by some commonly used antibodies such as anti-CEA. The specificity index of ¹²⁵I-PNA versus ¹³¹I-F(ab')₂ was actually found to decrease from 11.5 ± 1.1 at 24 hours to 8.4 ± 1.2 at 48 hours. A decrease of this localization index has been observed in many radioantibody investigations as the tumour mass becomes larger^{27, 252, 253, 266, 268, 270}. Larger tumours may contain necrotic tissue, which will no longer retain the specifically-binding protein²⁵³. In addition, if tumours above a certain critical size are rich in extravascular blood, they may show a much larger contribution of nonspecific macromolecular localization to the overall antibody localization mechanism²⁷⁰. The average mass of the RI tumours used in the present studies at 48 hours (1.47 grams), was about 50% higher than those tumours dissected at 24 hours (0.97 grams). Changes in (1) the relative ratios of viable to necrotic tissue, or (2) tumour vasculature, may have resulted in the lower

specificity indices observed at 48 hours.

Two other tissues examined in these biodistribution studies revealed significantly different specificity indices when comparing the two different animal tumour models. The high specificity index (19 ± 1.6) observed in the submaxillary glands of CBA/CAJ mice at 48 hours could either be due to localization of radioiodide or radiolabelled peanut lectin. The in vivo hydrolysis of the radioiodide from the proteins was found to be much more extensive for the radiolabelled peanut lectin than for the $F(ab')_2$ fragment. Analysis of plasma samples at 48 hours revealed that only 44 per cent of the peanut lectin radiolabel, ^{125}I , was precipitated by TCA while 75 per cent of the ^{131}I radioactivity was found to be protein bound in the plasma. Since it has been observed that large strain differences exist amongst mice in the ability of the thyroid to concentrate free iodide from the blood²⁷¹, perhaps this is also true of the uptake of free iodide in salivary glands. The selective localization of ^{125}I in the salivary glands may have also been due to PNA binding receptors in the salivary glands. Submaxillary gland mucin has been shown chemically to contain the T antigen^{162, 163}. Stoward et al. detected PNA reactive sites in mouse submandibular glands when they applied peanut lectin-horseradish peroxidase for histochemical staining of tissue sections. Perhaps some species variation accounted for the degree of unmasking of salivary gland glycoproteins, reactive with PNA, which

resulted in the high salivary gland specificity index.

The last tissue examined, which appeared to exhibit differential mouse tumour model distribution of PNA, was the spleen. The multiple functions of the spleen, and the presence of both lymphoid and reticuloendothelial cells²⁷² allow several alternative postulations for the specific PNA localization in the spleens of CBA/CAJ mice. The peanut lectin may be interacting with cells within the normal spleen of CBA/CAJ which have receptors for this lectin. Watanabe et al.²⁶⁰ demonstrated in vitro binding of PNA to intercellular fibrils at the peripheral region of the white pulp. Howard and Batsakis²⁷³ have recently proposed the potential usefulness of PNA as a marker of tissue histiocytes (macrophages). The major lymphoid mass of the spleen (white pulp) has easy access to the bloodstream, as it is concentrated around the arterioles of the spleen, in the form of periarterial sheaths²⁶¹. Several investigators have reported that between 5 and 6 per cent of spleen cells in CBA mice will bind PNA¹¹⁵⁻¹¹⁸ and there may be strain to strain variation in the extent of PNA receptor expression since it has been reported that 12 to 16% of the spleen cells of Balb/c mice bind PNA¹¹⁷. London et al.¹¹⁵ tested the distribution of PNA reactive cells in lymphoid organs of 3 different mouse strains and although only $2.7 \pm 1.2\%$ of the spleen cells in A/J strain bound the lectin, a very similar cell PNA-reactivity was found in spleens of CBA/J and C57BL/6 mice ($5.6 \pm 2.6\%$ and $6.5 \pm 3.1\%$, respectively),

It therefore seems unlikely that there would be preferential localization of PNA in the normal spleens of CBA/CAJ mice and not in the spleens of C57/BL mice.

Since one of the functions of the spleen is to remove particulates from the bloodstream²⁷², perhaps the peanut lectin protein was aggregated slightly in the one bio-distribution study and not the other. The storage of PNA at 4°C may have resulted in aggregation of the lectin since Decastel *et al.*⁸⁵ have found that peanut lectin is insoluble at low temperature and high concentrations (crybinsoluble). Storage at a concentration less than 0.8 mg/ml and in the presence of the saccharides, for which PNA has binding affinity, may help prevent cryoinsolubility⁸⁵. Although the possibility of some PNA aggregation may have contributed to some splenic uptake, the selective spleen uptake was not paralleled by a selective accumulation in the liver. There was no significant difference in the specificity indices of the livers of CBA/CAJ mice and C57BL mice.

It is interesting to note that the animal tumour model, in which the selective spleen uptake occurred, was the one bearing a tumour with PNA receptors. The presence of the tumour may have played an important role in the spleen localization. A number of other investigators have observed a much enhanced uptake of tumour-specific antibodies, as compared to non-specific antibodies, in the spleens of the tumour bearing mice^{274, 275}. Witz and coworkers²⁷⁴ found that an antibody, produced against a syngeneic mouse lymphoma, was

preferentially localized in the spleen and not in solid tumours. There was no such spleen fixation in normal mice. Pressman and Watanabe²⁷⁵ simultaneously injected ¹³¹I labelled antibodies (specific for a mouse plasma cell tumour), and ¹²⁵I-labelled normal globulin, into plasmacytoma-bearing mice. Calculation of the specificity indices from the data presented in their results reveals that while the specificity index was only 1.09 in the tumour, the corresponding value in the spleen was 8.6. Further in vivo purification of the antibody preparation actually resulted in less of the specific antibody preparation localizing in the tumour than the normal globulin and an increased value of 14.8 for the specificity index in the spleen²⁷⁵.

In the two above studies and the present PNA investigation, a possible explanation of the specific spleen localization, is the presence of tumour cells or antigens within the spleens of tumour-bearing mice. Lymphomas, such as the RI mouse tumour cell line, are known to metastasize readily to tissues such as the spleen^{276, 278}. One of the functions of the spleen, as a lymphoid organ, is the removal of foreign antigens and cells present in the bloodstream; thus, the spleen has excellent contact with the bloodstream²⁶¹. Tumour cells lodging within the spleen of the lymphoma bearing mice may therefore have better accessibility to tumour specific antibodies, and lectins, than to the tumour itself²⁷⁴. The tumour cells may have been selectively lodged in the spleen, in a manner similar to

the organotropic distribution of metastases which was described by Uhlenbruck⁵⁰. Both the liver and spleen are known to trap desialylated galactose-expressing cells such as N'RBC, by the action of tissue- and macrophage-lectins, specific for galactose^{194, 199}. On a per gram basis, the spleen has been found to be three times as efficient as the liver in trapping N'RBC²⁷⁹. Therefore, tumour cells with peanut lectin-receptors and galactose on their surface may also be selectively sequestered in the spleen, thereby resulting in the type of localization of radiolabelled peanut lectin which was evident in the present investigation.

Another hypothesis of the specific spleen localization of radiolabelled PNA is based upon the in vivo loss of T antigen which was observed in the solid RI tumour of CBA/CAJ mice, used in these studies. Many other tumour cell lines which have differential glycoprotein expression on their cell surfaces (depending on their growth environment) are known to shed soluble glycoproteins into serum^{263, 265}. If the RI losses surfaces glycoproteins during its growth in mice, intravenous injection of PNA may result in in vivo agglutination and/or precipitation and a subsequent specific localization in the spleen.

Further studies would be necessary to delineate the relationship between the in vivo growth of RI tumour cells and the associated tumour and splenic uptake of radiolabelled peanut lectin. The possibility of metastatic

localization and detection with radiolabelled peanut lectin appears particularly exciting since Springer et al.^{66, 151} found metastatic human cancer lesions persisted in the expression of T antigen receptors.

SUMMARY AND CONCLUSIONS

- 1) An RI mouse lymphoma cell line, shown to express T-like antigen receptors, was successfully grown in vivo producing an animal tumour model for the investigation of the in vivo localizing capability of T antigen-avid proteins.
- 2) In preliminary experiments, anti-T antibodies were radiolabelled in a protected form to provide a radioiodinated protein which bound avidly to a synthetic T immunoadsorbent. A high binding affinity and specificity of radiolabelled anti-T for neuraminidase-treated red blood cells or RI tumour cells was not demonstrable. In vivo biodistribution studies of ^{125}I anti-T antibodies indicated that the extent of anti-T tumour localization could be matched by that of a nonspecific gamma globulin.
- 3) An affinity purified peanut lectin preparation was radioiodinated and desalted by gel filtration to provide a radiolabelled protein which maintained in excess of 95% of its initial radiochemical purity, when stored at 4 C for two weeks.
- 4) Radiolabelled peanut lectin exhibited a very specific and avid in vitro binding for T-like cell surface glycoproteins in both RI tumour cells and neuraminidase-treated red blood cells (N'RBC). Radioiodinated preparations of PNA bound, on average, 66% to a one ml suspension of 2% N'RBC and 20% to 5×10^6 RI tumour cells. The binding was readily inhibited in the

- presence of galactose and could not be demonstrated with control cells, lacking the T antigen.
- 5) The carbohydrate-binding specificity of ^{125}I -PNA was investigated, using synthetic carbohydrate immuno-adsorbents. The lectin was found to have an avidity for the T disaccharide, β -D-Gal(1 \rightarrow 3)-GalNAc which was about 50-fold (α -linked), and 40-fold (β -linked) higher than for galactose and at least 10-fold higher than that seen for all other carbohydrates tested.
 - 6) ^{125}I Peanut lectin was found to have good tumour localization and rapid blood clearance, upon i.v. injection in CBA/CAJ mice, bearing RI tumours. Clear images of tumours were obtained in serial scintigraphic imaging by 24 and 48 hours post-injection. No blood subtraction techniques were necessary for tumour delineation.
 - 7) Biodistribution studies of ^{125}I -PNA in RI tumour-bearing CBA/CAJ mice, revealed tumour:background tissue ratios of radioactivity which increased steadily with time. The tumour:blood and tumour:muscle ratios were 17:1 and 40:1, respectively, at 48 hours.
 - 8) The in vivo specificity of retention of radioiodinated peanut lectin in RI tumours was verified when ^{125}I -PNA tumour retention was eleven fold higher than that found with a nonspecific protein $\text{F}(\text{ab}')_2$ fragment of IgG. Such preferential tumour uptake was not evident

in an EL4 mouse tumour model, in which the tumour did not express PNA receptors.

- 9) A significant spleen uptake of radiolabelled peanut lectin occurred in mice, bearing RI tumours, but was not evident in mice bearing EL4 tumours.
- 10) Based on the data from in vitro binding investigations and in vivo biodistribution studies in tumour-bearing mice, radioiodinated peanut lectin appears to offer a potential utility in the radioimmunodetection of tumours expressing the T antigen.

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APPENDIX I: COMPARATIVE TISSUE RATIOS OF RADIOACTIVITY OF
I-125 ANTI-T ANTIBODY AND I-131 NORMAL GAMMA GLOBULIN
IN RI LYMPHOMA-BEARING CBA/CAJ MICE

		8HR(N=6)		24HR(N=6)		48HR(N=6)	
		T-AB	IGG	T-AB	IGG	T-AB	IGG
BLOOD	ORGAN/BLOOD	1.00	1.00	1.00	1.00	1.00	1.00
	STAN. DEV.	0.0	0.0	0.0	0.0	0.0	0.0
	ORGAN/MUSCLE	18.47	20.60	10.64	11.87	9.65	10.34
	STAN. DEV.	3.42	3.05	1.70	2.17	3.05	3.36
LIVER	ORGAN/BLOOD	0.28	0.21	0.31	0.23	0.30	0.22
	STAN. DEV.	0.02	0.01	0.01	0.02	0.03	0.02
	ORGAN/MUSCLE	5.14	4.24	3.28	2.76	2.78	2.20
	STAN. DEV.	1.12	0.64	0.56	0.48	0.70	0.63
SPLEEN	ORGAN/BLOOD	0.26	0.22	0.27	0.24	0.30	0.26
	STAN. DEV.	0.06	0.04	0.04	0.03	0.06	0.04
	ORGAN/MUSCLE	4.86	4.68	2.89	2.85	2.84	2.69
	STAN. DEV.	1.78	1.35	0.71	0.71	0.93	0.88
KIDNEY	ORGAN/BLOOD	0.39	0.30	0.49	0.36	0.45	0.34
	STAN. DEV.	0.04	0.02	0.08	0.06	0.07	0.04
	ORGAN/MUSCLE	7.21	6.22	5.28	4.35	4.21	3.38
	STAN. DEV.	1.24	0.93	1.56	1.30	0.92	0.86
TUMOUR	ORGAN/BLOOD	0.40	0.36	0.50	0.45	0.49	0.44
	STAN. DEV.	0.08	0.07	0.08	0.07	0.07	0.09
	ORGAN/MUSCLE	7.44	7.51	5.43	5.41	4.87	4.73
	STAN. DEV.	2.38	2.42	1.51	1.72	1.98	2.09
MUSCLE	ORGAN/BLOOD	0.06	0.05	0.10	0.09	0.12	0.11
	STAN. DEV.	0.01	0.01	0.02	0.02	0.05	0.05
	ORGAN/MUSCLE	1.00	1.00	1.00	1.00	1.00	1.00
	STAN. DEV.	0.0	0.0	0.0	0.0	0.0	0.0

APPENDIX II TISSUE RATIOS OF RADIOACTIVITY OF I-125 PEANUT LECTIN
IN CBA/CAJ MICE BEARING RI LYMPHOMA TUMOURS

	ORGAN TO BLOOD RATIOS				ORGAN TO MUSCLE RATIOS				
	3HR N=7	8HR N=6	24HR N=8	48HR N=8	3HR N=7	8HR N=6	24HR N=8	48HR N=8	
BLOOD	ORGAN/BLOOD STAN. DEV.	1.00 0.0	1.00 0.0	1.00 0.0	ORGAN/MUSCLE STAN. DEV.	4.67 1.24	7.13 2.54	5.59 1.59	2.30 0.57
LIVER	ORGAN/BLOOD STAN. DEV.	0.50 0.06	0.52 0.12	1.26 0.41	ORGAN/MUSCLE STAN. DEV.	2.26 0.39	3.72 1.62	6.87 2.23	14.06 4.08
SPLEEN	ORGAN/BLOOD STAN. DEV.	3.30 0.88	3.92 2.13	6.90 2.24	ORGAN/MUSCLE STAN. DEV.	14.62 2.21	27.80 17.16	37.43 11.58	41.58 13.20
KIDNEY	ORGAN/BLOOD STAN. DEV.	5.78 1.35	5.06 1.94	8.40 2.90	ORGAN/MUSCLE STAN. DEV.	26.62 9.70	36.67 22.75	46.02 17.07	70.21 21.45
STOMACH	ORGAN/BLOOD STAN. DEV.	6.03 1.22	8.88 1.34	8.04 1.40	ORGAN/MUSCLE STAN. DEV.	27.58 7.69	63.05 22.08	44.69 14.80	15.55 5.20
PANCREAS	ORGAN/BLOOD STAN. DEV.	0.66 0.14	0.62 0.10	0.48 0.08	ORGAN/MUSCLE STAN. DEV.	2.97 0.62	4.30 1.36	2.64 0.88	1.88 0.73
DUODENUM	ORGAN/BLOOD STAN. DEV.	1.68 0.64	1.98 0.78	1.29 0.61	ORGAN/MUSCLE STAN. DEV.	7.40 1.93	14.07 6.42	6.88 3.03	3.77 1.06
GIT	ORGAN/BLOOD STAN. DEV.	0.95 0.16	0.96 0.16	0.70 0.13	ORGAN/MUSCLE STAN. DEV.	4.30 0.63	6.92 2.91	3.79 0.82	2.71 0.91
MUSCLE	ORGAN/BLOOD STAN. DEV.	0.23 0.07	0.15 0.04	0.19 0.06	ORGAN/MUSCLE STAN. DEV.	1.00 0.0	1.00 0.0	1.00 0.0	1.00 0.0
TUMOUR	ORGAN/BLOOD STAN. DEV.	2.57 0.61	3.30 1.82	5.79 3.17	ORGAN/MUSCLE STAN. DEV.	11.54 2.62	22.44 10.66	33.60 20.80	39.80 17.59

APPENDIX III LOCALIZATION INDEX OF PEANUT LECTIN

Tissue:Blood ¹²⁵I-Peanut Lectin
 Tissue:Blood ¹²⁵I-F(ab')₂ IgG

Tissue	24 hr		48 hr	
	RI Tumour CBA/CAJ Mice n=4	EL4 Tumour C57/B1 Mice n=6	RI Tumour CBA/CAJ Mice n=5	EL4 Tumour CBA/CAJ Mic n=4
Tumour	11.5 ± 1.1	1.3 ± 0.1	8.4 ± 1.2	1.6 ± 0.5
Muscle	2.5 ± 0.3	2.4 ± 0.3	2.7 ± 0.2	2.5 ± 0.7
Liver	4.1 ± 0.4	3.4 ± 0.3	5.4 ± 0.4	6.8 ± 1.3
Kidney	6.1 ± 0.4	6.3 ± 0.8	6.6 ± 0.3	9.9 ± 2.0
Stomach	2.9 ± 0.3	1.9 ± 0.5	3.4 ± 0.3	2.8 ± 0.4
GIT	2.4 ± 0.3	2.1 ± 0.1	2.7 ± 0.2	2.9 ± 0.2
Salivary	7.6 ± 1.1	3.0 ± 0.7	19.0 ± 1.6	3.5 ± 0.9
Sex Organs	3.6 ± 1.5	2.3 ± 0.4	6.2 ± 0.6	4.9 ± 1.8
Thyroid	3.4 ± 0.9	4.4 ± 2.5	3.1 ± 1.1	3.8 ± 2.8
Spleen	25.6 ± 2.7	6.2 ± 0.4	27.0 ± 2.0	8.7 ± 2.3
Lung	3.6 ± 1.5	2.9 ± 0.3	4.4 ± 0.3	4.6 ± 1.4
Thymus	2.0 ± 0.6	-	2.1 ± 0.3	-

APPENDIX V
 COMPARATIVE BIODISTRIBUTION OF I-125 PEANUT LECTIN AND I-131 F(AB')₂
 PERCENT INJECTED DOSE INCORPORATED PER ENTIRE ORGAN

	CBA/CAJ MICE BEARING RI TUMOURS						C57/BL MICE BEARING EL4 TUMOURS							
	8HR(N=3)		24HR(N=4)		48HR(N=5)		72HR(N=1)		24HR(N=6)		48HR(N=4)			
	PNA	FAB	PNA	FAB	PNA	FAB	PNA	FAB	PNA	FAB	PNA	FAB		
BLOOD	\bar{X}	1.88	8.13	0.30	1.35	0.11	0.39	0.04	0.16	\bar{X}	0.50	2.46	0.13	0.57
	SD	0.48	0.86	0.03	0.17	0.01	0.05	0.0	0.0	SD	0.11	0.65	0.04	0.08
LIVER	\bar{X}	1.24	2.69	0.45	0.52	0.32	0.23	0.25	0.14	\bar{X}	0.48	0.71	0.26	0.19
	SD	0.18	0.51	0.06	0.16	0.01	0.02	0.0	0.0	SD	0.05	0.20	0.05	0.02
SPLEEN	\bar{X}	0.79	0.19	0.19	0.03	0.09	0.01	0.04	0.00	\bar{X}	0.10	0.08	0.03	0.02
	SD	0.02	0.04	0.02	0.00	0.01	0.00	0.0	0.0	SD	0.03	0.04	0.00	0.00
KIDNEY	\bar{X}	4.48	4.48	0.96	0.71	0.42	0.26	0.25	0.14	\bar{X}	0.91	0.74	0.36	0.17
	SD	0.69	0.73	0.04	0.04	0.03	0.01	0.0	0.0	SD	0.15	0.26	0.11	0.01
STOMACH	\bar{X}	3.08	4.97	0.69	1.11	0.11	0.13	0.05	0.04	\bar{X}	0.27	0.71	0.12	0.18
	SD	0.67	1.08	0.18	0.44	0.05	0.06	0.0	0.0	SD	0.11	0.28	0.07	0.07
GIT	\bar{X}	4.01	5.63	0.50	0.94	0.16	0.24	0.08	0.10	\bar{X}	0.60	1.42	0.16	0.26
	SD	0.66	0.54	0.14	0.20	0.01	0.01	0.0	0.0	SD	0.15	0.56	0.04	0.04
SALIVARY	\bar{X}	1.10	1.47	0.23	0.14	0.20	0.04	0.14	0.01	\bar{X}	0.10	0.15	0.02	0.02
	SD	0.56	0.84	0.02	0.03	0.02	0.01	0.0	0.0	SD	0.07	0.06	0.02	0.01
MUSCLE	\bar{X}	0.16	0.28	0.02	0.04	0.00	0.01	0.00	0.00	\bar{X}	0.02	0.06	0.01	0.01
	SD	0.03	0.05	0.00	0.00	0.00	0.00	0.0	0.0	SD	0.00	0.03	0.00	0.00
TUMOUR	\bar{X}	1.33	0.82	1.21	0.48	0.49	0.22	0.22	0.12	\bar{X}	0.50	1.82	0.24	0.65
	SD	1.06	0.27	0.28	0.05	0.25	0.08	0.0	0.0	SD	0.27	0.97	0.29	0.66
LUNG	\bar{X}	0.33	0.41	0.09	0.13	0.03	0.03	0.01	0.00	\bar{X}	0.08	0.14	0.03	0.04
	SD	0.03	0.07	0.02	0.12	0.00	0.00	0.0	0.0	SD	0.02	0.04	0.01	0.01
SEX	\bar{X}	0.79	1.05	0.08	0.10	0.05	0.03	0.97	1.24	\bar{X}	0.06	0.11	0.02	0.01
	SD	0.35	0.42	0.04	0.06	0.00	0.00	0.0	0.0	SD	0.04	0.07	0.01	0.01
THYROID	\bar{X}	0.67	0.92	0.37	0.48	0.46	0.60	0.00	0.00	\bar{X}	0.53	0.61	0.15	0.19
	SD	0.24	0.31	0.13	0.16	0.24	0.32	0.0	0.0	SD	0.56	0.62	0.15	0.18
THYMUS	\bar{X}	0.02	0.03	0.00	0.01	0.00	0.00	0.0	0.0	\bar{X}	0.00	0.00	0.00	0.00
	SD	0.02	0.03	0.00	0.00	0.00	0.00	0.0	0.0	SD	0.00	0.00	0.00	0.00

APPENDIX IV COMPARATIVE BIODISTRIBUTION OF I-125 PEANUT LECTIN AND I-131 F(AB')
PERCENT OF INJECTED DOSE INCORPORATED PER GRAM TISSUE

TUMOUR	CBA/CAJ MICE BEARING RI TUMOURS						C57/BL MICE BEARING EL4 TUMOURS							
	8HR(N=3)		24HR(N=4)		48HR(N=5)		72HR(N=1)		24HR(N=6)		48HR(N=4)			
	PNA	FAB	PNA	FAB	PNA	FAB	PNA	FAB	PNA	FAB	PNA	FAB		
TUMOUR	\bar{X}	4.80	3.32	1.30	0.52	0.33	0.16	0.16	0.092	\bar{X}	0.34	1.27	0.18	0.50
	SD	2.53	0.17	0.18	0.04	0.09	0.03	0.0	0.0	SD	0.08	0.34	0.15	0.29
BLOOD	\bar{X}	1.38	6.00	0.20	0.89	0.06	0.26	0.03	0.114	\bar{X}	0.39	1.92	0.12	0.53
	SD	0.30	0.41	0.02	0.09	0.00	0.02	0.0	0.0	SD	0.06	0.45	0.04	0.09
LIVER	\bar{X}	1.07	2.31	0.35	0.40	0.24	0.18	0.21	0.129	\bar{X}	0.43	0.62	0.29	0.21
	SD	0.08	0.25	0.03	0.10	0.02	0.02	0.0	0.0	SD	0.02	0.14	0.09	0.05
SPLEEN	\bar{X}	9.24	2.20	1.92	0.34	0.88	0.13	0.62	0.083	\bar{X}	0.76	0.62	0.26	0.15
	SD	1.57	0.12	0.24	0.04	0.03	0.02	0.0	0.0	SD	0.06	0.15	0.08	0.03
KIDNEY	\bar{X}	3.00	13.01	2.69	2.00	1.21	0.74	0.82	0.481	\bar{X}	4.28	3.44	2.09	0.99
	SD	0.50	1.05	0.15	0.11	0.11	0.06	0.0	0.0	SD	0.96	1.07	0.75	0.08
STOMACH	\bar{X}	9.73	15.69	1.23	1.93	0.38	0.45	0.12	0.091	\bar{X}	1.18	3.17	0.44	0.72
	SD	3.15	5.05	0.28	0.28	0.05	0.05	0.0	0.0	SD	0.41	1.36	0.18	0.23
GIT	\bar{X}	1.34	1.88	0.15	0.29	0.06	0.09	0.03	0.048	\bar{X}	0.25	0.60	0.10	0.16
	SD	0.18	0.12	0.03	0.05	0.00	0.01	0.0	0.0	SD	0.04	0.18	0.02	0.04
SALIVARY	\bar{X}	6.24	8.24	1.51	0.90	1.30	0.28	1.13	0.074	\bar{X}	0.78	1.23	0.20	0.26
	SD	2.02	3.24	0.21	0.21	0.08	0.03	0.0	0.0	SD	0.49	0.36	0.14	0.07
LUNG	\bar{X}	3.06	3.79	0.78	0.96	0.25	0.23	0.10	0.083	\bar{X}	0.59	1.01	0.25	0.28
	SD	0.28	0.24	0.04	0.71	0.03	0.03	0.0	0.0	SD	0.14	0.30	0.08	0.09
SEX	\bar{X}	3.17	4.21	0.39	0.49	0.18	0.11194	4.1248	6.41	\bar{X}	0.33	0.71	0.23	0.25
	SD	1.01	1.03	0.07	0.19	0.02	0.02	0.0	0.0	SD	0.04	0.26	0.09	0.15
THYMUS	\bar{X}	3.10	5.46	0.68	1.52	0.25	0.49	0.0	0.0	\bar{X}	3.16	5.72	0.22	0.64
	SD	3.16	5.72	0.22	0.64	0.06	0.10	0.0	0.0	SD	0.06	0.10	0.0	0.0