1991

Immunolocalization and a study of adhesive properties of PAS IV

Sheetal A. Pandya
San Jose State University

Follow this and additional works at: https://scholarworks.sjsu.edu/etd_theses

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Immunolocalization and a study of adhesive properties of
PAS IV

Pandya, Sheetal A., M.S.

San Jose State University, 1991

IMMUNOLOCALIZATION AND A STUDY
OF
ADHESIVE PROPERTIES OF PAS IV

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the degree
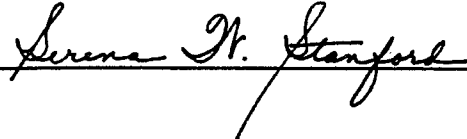
Master of Science

By

Sheetal A. Pandya

May, 1991

APPROVED FOR THE DEPARTMENT OF CHEMISTRY

_____

Dr. Dale Greenwalt

_____

Dr. Gerald Selter

_____

Dr. Leon Yengoyan

APPROVED FOR THE UNIVERSITY

_____

ABSTRACT

IMMUNOLOCALIZATION AND A STUDY OF ADHESIVE PROPERTIES OF PAS IV

by Sheetal A. Pandya

The presence of milk fat globule membrane glycoprotein, PAS IV, on human and bovine tissue sections was studied by immunolocalization. The ABC technique was used for the study. Presence of PAS IV was observed on bovine lactating mammary tissue sections and human normal breast tissue sections. PAS IV was detected on the capillaries of human breast tumor sections. The rest of the tissue section did not show any presence of PAS IV. Antibodies against PAS IV recognize a platelet membrane glycoprotein, GP IV. Comparison of hydrophobicity, N-terminal amino acid sequence and purification procedures suggest similarity between PAS IV and GP IV. GP IV is a receptor for collagen and thrombospondin on the platelet surface. GP IV also binds C1q. Adhesive nature of PAS IV was studied. It was observed that PAS IV does not bind collagen, thrombospondin and C1q. The results suggest PAS IV and GP IV may have different functions to perform in their resident cells.

ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 : INTRODUCTION

1.1) MEMBRANES AND PROTEINS

All living cells are surrounded by asymmetric sheet like structures called membranes. These sheet like structures are noncovalent assemblies that are thermodynamically stable but metabolically active. By forming closed compartments around cellular protoplasm, (plasma) membranes impart cellular individuality to the cells. They act as barriers with selective permeabilities to information and material. Membranes also form distinguishable organelles within the cell such as mitochondria, golgi complexes, endoplasmic reticulum and nuclear membrane. Most membranes are composed predominantly of lipids and proteins but also contain some carbohydrates in the form of glycoproteins. Specific protein molecules are anchored in lipid bilayers where they carry out specific functions of the orgenelle, cells and the organism (1).

Depending upon their orientation in membranes, proteins are classified as either integral or peripheral. Integral proteins consist of hydrophilic segments separated by intervening hydrophobic regions which interact with the hydrophobic interior of the membrane. Nonionic detergents extract integral proteins, without loss of function in most cases, since the hydrophilic ends of the proteins are not bound by the detergent. However, ionic detergents interact with both the hydrophilic ends and the hydrophobic portion of the integral proteins and inactivate their function (1, 2).

Integral membrane proteins are not extracted by treatment with 0.1M Na_2CO_3, a treatment known to extract peripheral proteins from the membrane (11). As the term suggests, peripheral proteins are weakly bound to the periphery of the membrane. They do not penetrate the hydrophobic interior of the membrane (2, 3, 7).

1

Integral proteins along with other components of the membrane, provide selective permeability to the membrane and are responsible for the transport of the permeable material. Peripheral proteins may act as specific receptors for enzymes etc. (1, 2).

1.2) MILK FAT GLOBULE MEMBRANE (MFGM)

The nature of MFGM is best understood with some knowledge of the globule's origin. A fundamental unit of structure in lactating mammary tissue is the alveolus, a sphere shaped arrangement of lactating cells around a lumen. The actual synthesis of the milk fat globule takes place in the lactating cells. Fat droplets form in the cell and ultimately move to the apical region where they become enveloped in plasma membrane and are expelled from the cell into the lumen. The milk fat globule is. therefore, essentially an oil droplet enclosed in plasma membrane from the lactating cell. The unique secretory process provides the membranologist with a source of plasma membrane on an inert (glyceride) core with the same exterior exposure it had on the cell. MFGM can be obtained easily from milk in substantial quantity and purity by relatively mild manipulations (5, 6, 7).

Early analyses have shown that MFGM and the plasma membrane from bovine mammary gland have similar electrophoretic patterns (5). Seven periodic acid/ Schiff (PAS) - positive bands have been detected after separation of MFGM by SDS polyacrylamide gel electrophoresis. These proteins have been numbered I to VII. Two of them, butyrophilin and xanthine oxidase, account for 50% of the total protein. PAS IV , a glycoprotein, constitutes about 5% of MFGM proteins (7, 8, 9).

2

1.3) PAS IV

PAS IV (Bovine and Human) is a glycoprotein which is thought to span the membrane phospholipid bilayer in a variety of cells. It is associated with secretory epithelial cells and capillary endothelial cells present in heart, liver, spleen, pancreas, salivary glands and small intestine. As described above, it is also associated with the milk fat globule membrane. from which it has been purified (1, 2). Thus, PAS IV is associated with normal lactating mammary cells (8).

PAS IV is very hydrophobic, consisting of 50% nonpolar amino acids. Another 20% of the amino acids have side chains with uncharged polar groups at neutral pH. A high percentage of branched chain amino acids (valine, leucine and isoleucine) has been reported (10). During isolation from MFGM, PAS IV is recovered in the detergent phase of the nonionic detergent TX-114, a phase in which hydrophobic proteins are recovered (11). PAS IV is resistant to proteolysis when associated with the membrane. All these observations suggest that PAS IV is closely associated with the hydrophobic core of the membrane and therefore is an integral membrane protein (11).

Similarities between PAS IV from different sources have been studied. Human PAS IV and guinea-pig PAS IV have a molecular weight of 80,000 Da. Bovine PAS IV has a molecular weight of 78,000 Da. Other characteristics as described above are also common to PAS IV from these different sources. These proteins may have specific functions related to milk secretion in mammary tissue (9,11).

1.4) GP IV

Immunoblotting studies have shown that affinity purified antibody to human PAS IV recognized bovine PAS IV and another protein, GP IV, as

3

TABLE 1 : Comparison of some properties[+] of PAS IV and GP IV.

PROPERTIES	PAS IV	GP IV	REFERENCES
Molecular Weight	Bovine 78,000 Da Human 80,000 Da	Human 88,000 Da	8, 11, 17, 32
Nature	hydrophobic (based on its amino acid content)	hydrophobic (based on its amino acid content)	8, 11, 17
Extraction with TX-114	recovered in the detergent phase	recovered in the detergent phase	8, 11, 17, 32
Action of proteases	resistant when present on intact membrane	resistant when present on intact platelets	8, 12
Antibody cross reactivity	anti GP IV recognizes PAS IV	anti PAS IV recognizes GP IV	32
Isoelectric point	pH range of 7.8 to 8.5	pH range of 4.4 to 6.3	8, 11, 12, 17
Percent carbohydrate	30%	25% (17) 40% (25)	12, 17, 32, 25
Carbohydrate linkage	N-linked	N and O-linked	8, 17
Sialic acid content	3.65 mole/mole of protein	25.5 mole/mole of protein	8, 17

† N-terminal sequence homology between PAS IV and GP IV.

Human PAS IV : SXDXNSGLIAGAVIGAVLAVFGGILMPVG.

GP IV : GXDRNXGLIAGAVIGAVLAVFGGILMPVGDLPXQKF.

Bovine PAS IV : GVNRNSGLIAGAVIGAVLAVFGGILMPV.

4

well (32). GP IV is a glycoprotein, identified and isolated from platelet membranes. Apart from platelets, GP IV is also localized on endothelial cells, monocytes and some tumor cell lines including the C32 melanoma cell line (12, 17, 18). GP IV is a single, highly glycosylated polypeptide chain with a molecular weight of 88,000 Da. It has been reported that amino acids comprise 74% of the total mass of GP IV (17). The carbohydrate content would therefore be 26%. A cDNA clone encoding GP IV synthesizes a polypeptide with 471 amino acid residues. The predicted molecular weight of the polypeptide is about 53,000 Da. Assuming that the rest of the weight in the 88,000 Da GP IV is due to carbohydrates, the carbohydrate content is calculated as 40% (16). The two reported observations about the carbohydrate content in GP IV, thus, differ from each other. GP IV contains a high percent of nonpolar amino acids and is hydrophobic. When present on intact platelets, GP IV is resistant to proteolytic enzymes (12, 15, 17).

A comparison of some of the properties of GP IV and PAS IV is summarized in Table 1.

GP IV functions as a thrombospondin receptor on the platelet surface (12). It is capable of binding collagen fibrils (8) and erythrocytes infected with mature *Plasmodium falciparum* parasites (16). The importance of these interactions and role of GP IV may best be explained after a discussion of the basal lamina and its interactions with adhesive macromolecules.

1.5) BASAL LAMINAE

Basal laminae are thin layers of specialized extracellular matrix that underlie all epithelial cells. The matrix separates cells from the underlying or surrounding connective tissue. Basal laminae show a wide diversity in their functions. They help to regulate the passage of

macromolecules from the blood into the surrounding tissue. Basal laminae also act as a selective cellular barrier. Various studies suggest that specific components in the extracellular matrix may play a vital role in cell recognition processes including those involved in embryonic development and cancer cell metastasis (3).

1.6) ADHESIVE MACROMOLECULES

1.6.1) COLLAGEN

Collagen, the major macromolecule of connective tissues, is the most abundant protein in the animal world. It provides the extracellular framework for all animals and exists in virtually every animal tissue. It has been observed that collagen substrates alter the morphology, migration and adhesion of cells and, in some cases, regulate the differentiation of cultured cells (4). Recent studies suggest that specific glycoproteins bind cells to collagen (3, 4).

To date, 12 different types of collagen termed as type I, II, III, IV, V etc., in mammalian tissue have been identified. Type IV collagen is most abundant in the basal lamina (3,4). Thus collagen exists as a family of molecules sharing many properties. Three polypepetide subunits coil together to form a triple helix. Three of these left handed helices are then wound in a right handed superhelix to form a stiff rod like molecule. These helical molecules are then associated to form typical collagen fibrils. The collagen triple helix is stabilized by multiple interchain crosslinks between lysyl and hydroxylysyl residues among other crosslinks. Mature collagen is a glycoprotein containing oligosaccharides attached in 0-glycosidic linkage to the hydroxylysine residues. Different cells bind to specific types of collagen. Epithelial cells bind preferentially

to type IV collagen. Cells like breast epithelial cells are maintained long-
er on collagenous substrates than on plastic (4).

1.6.2) THROMBOSPONDIN (TSP)

Thrombospondin is a 450,000 Da glycoprotein composed of three iden-
tical disulfide linked chains with globular regions forming both terminal
ends. Thrombospondin has been detected on platelet membranes where
it is one of the factors which mediates platelet aggregation (12). Throm-
bospondin has been immunolocalized on peritubular connective tissue of
human kidney sections, dermal epidermal junctions and sweat glands of
skin, glandular areas of embryonic lung, interstitial areas of skeletal mus-
cle and the subendothelial region of aorta (13, 14). It is synthesized by
endothelial cells, fibroblasts, smooth muscle cells, alveolar pnuemocytes,
monocytes, nueroglial cells and is thought to be incorporated into the ma-
trix of these cells (12).

Thrombospondin is also found in high concentrations in colostrum and
some breast cyst fluids. Colostrum, the milk secreted by mammary cells
at the initiation of lactation, contains about 70-80 ug/ml of thrombospon-
din. Concentrations of thrombospondin drop as lactation becomes estab-
lished. Thrombospondin has been isolated from colostrum as well as
platelets (13, 19).

1.7) CELL MATRIX INTERACTIONS AND THE ROLE OF ADHESIVE MACROMOLECULES

Adhesive macromolecules play a central role in cell-cell and cell-sub-
strate interaction. They provide a framework upon which cell migration
and morphogenesis occur during development. Although some molecules
are intrinsic to the membrane, most are ligands for cellular receptors (4,
12). Platelet aggregation is a useful example for characterizing cell adhe-

7

sion phenomena. A break in the blood vessel wall will expose platelets to the collagen present in the surrounding basal matrix (4, 23, 24). This stimulates the alpha granules in platelets to secrete numerous adhesive proteins. These proteins bind to their specific receptors on the platelets and mediate platelet aggregation. One such protein secreted by the alpha granules is thrombospondin and GP IV has been shown to be its receptor on the platelet surface (12, 18). GP IV, therefore may be responsible for mediating platelet aggregation.

Another example of cell cell interaction is adherence of *Plasmodium falciparum* infected erythrocytes. Erythrocytes infected with *Plasmodium falciparum* express knob like adhesion structures that allow the infected cells to cling to the capillary endothelium of host organs (25). This contributes to the survival of the parasite by protecting the infected erythrocytes from destruction in the spleen (16). GP IV has been shown to be the receptor for binding of parasitized erythrocytes on the endothelial cells of the blood vessels. In addition, GP IV on platelets can mediate binding of platelets to the infected erythrocytes and block immunological reaction by the host (16, 25).

Cell matrix interactions constitute an important factor in maintaining the viability of the connective tissue, particularly during tissue remodeling and wound healing (20). Another such interaction is involved in tumor cell metastasis. When a cell from a tumor enters the blood stream, it needs to penetrate the basal lamina and come in contact with the outlining endothelial cells of the blood vessel, to enter another tissue. This process is called diapedesis and is part of the metastatic migration of tumor cells (20, 4).

Adhesive macromolecules and adhesive protein receptors probably have a major role to play in this process. An adhesive protein receptor for the components of the basal laminae would facilitate this process. GP

IV functions as an adhesive protein receptor on the platelet membrane (8,12,16). PAS IV is similar to GP IV (32). The present study attempts to explore the possibility of PAS IV being an adhesive protein receptor.

If PAS IV is present on human breast tumor cells and is able to bind collagen and thrombospondin, it may be responsible for mediating diapedesis of these cells. There are three mechanisms by which diapedesis could be mediated by PAS IV. These mechanisms described below, are theorized and yet to be proved.

Collagen is abundantly present in basal lamina (4). A break in the endothelium lining of the wall of the blood vessel would expose the blood components, like a circulating tumor cell, to collagen of the matrix. If PAS IV on the tumor cell could bind to the collagen and mediate adherence of the tumor cell to the matrix, the tumor cell would be able to penetrate the matrix and proliferate in that tissue.

GP IV binds thrombospondin. Assuming PAS IV also could bind thrombospondin, we can postulate a second mechanism for diapedesis. Thrombospondin can act as a bridge between GP IV on the endothelial cells of the blood vessel and PAS IV on the tumor cell. Once the tumor cell is adhered to the blood cell wall, it can move between the cells and penetrate the basal laminae.

Thrombospondin is also present in the basal lamina. PAS IV could directly bind to thrombospondin in a similar manner to collagen and mediate diapedesis.

1.8) FIRST COMPONENT OF COMPLEMENT C1Q

GP IV has also been shown to bind C1q (personal communication, G. Jamieson). C1q, present in the blood, is a part of the complement system which activates the immunological response of the body. The comple-

ment system is responsible for the destruction and elimination of foreign material from the body, notably bacteria and viruses. Complement may also be activated by complex polysaccharides such as those in yeast or bacterial cell walls before reaction with antibody and hence it can form an immediate defense against infection before immunity sets in. C1 is the first component of the complement system and itself has three subunits. C1q, C1r, C1s. C1 comes in contact with the antigen-antibody aggregate and becomes activated. The interaction of C1 with aggregated antibody is through C1q which binds to the second constant region of immunoglobulin G (IgG). C1q in turn activates other components of the complement. The terminal components C5 and C9 are responsible for membrane damage of the foreign organism (26, 27). C1q is a 400 kDa protein with three types of chains very similar to each other. These chains give C1q a unique structure which is half globular and half collagen like (26).

CHAPTER 2 : MATERIALS AND METHODS

2.1) MATERIALS

Tris base, tris-HCl, Triton X-100, sodium dodecyl sulphate (SDS), gly-cine (freebase anhydrous), bovine serum albumin, fibrinogen, collagen (calf skin), gelatin, diaminobenzidine (DAB), 2,2' azino-bis(3-ethyl benz-thiozoline-6-sulfonic acid) (ABTS), Y globulins (bovine cohn fraction) and cyanogen bromide activated Sepharose 4B were purchased from Sigma Chemical Co. (St. Louis, MO). Bis (N, N'- methylene- bis- acrylamide), affinity purified goat anti-rabbit (whole molecule) horse radish peroxidase conjugate and coomaise brilliant blue R-250 were purchased from Bio-Rad (Richmond, CA). Acrylamide and ammonium persulfate were pur-chased from Spectrum Chemical MFG Corp. (Redondo Beach, CA). Bicinchoninic acid (BCA) and Tween 20 were purchased from Pierce Chemical CO (Rockford, IL). Gel bond was purchased from the FMC Corporation (Rockland, ME). Gel gard 2500 film was purchased from Hoechest Celanese (Charlotte, NC). 2-Mercaptoethanol was purchased from Aldrich Chemical Company (Mllwaukee, WI). Centricon 30 micro-concentrators were purchased from Amicon Division, W. R. Grace & Co., (Danvers, MA). Avidin Biotin Complex (ABC) kit was purchased from Vector Laboratories (Birmingham, CA). Tissue section slides were purchased from Tissue Procurement Service, University of Alabama, Bur-lingham, AL.

2.2) METHODS

2.2.1) PURIFICATION OF IMMUNOGLOBULINS FROM RABBIT WHOLE SERUM

Immunoglobulins G (IgG) from preimmunized rabbit serum were affinity purified to be used as negative control antibodies. A 1:1 solution by volume of whole serum was prepared with 20 mM Tris buffered saline (150 mM) pH 7.4 (TBS). A saturated solution of ammonium sulfate (40% after dissolving) was added drop by drop with constant stirring to precipitate the IgG. The stirring was continued for 45 minutes. The precipitated proteins were centrifuged at 10,000 g for 20 minutes. The pellet was redissolved in 3 ml of TBS and dialyzed against a 100 fold volume of TBS overnight. The dialyzed preparation was concentrated using a Centricon filter. The sample was then run through a Protein A affinity column. The 3 ml Protein A column was equilibrated with TBS before use. After loading 15 ml of the sample, the column was again washed with 30 ml of 100 mM Tris buffered saline pH 8.0 and 30 ml of 10 mM Tris buffered saline pH 8.0 successively to remove the unbound material. The flow rate was kept constant at 18 ml/hour. Immunoglobulins were eluted with 15 ml of 100 mM glycine pH 2.5. Three milliliter fractions were collected in tubes containing 100 ul of 1 M Tris buffered saline pH 8.0 to neutralize the low eluting pH. Eluted fractions were subjected to SDS polyacrylamide gel electrophoresis to determine the fractions containing the immunoglobulins. These fractions were then combined together and a BCA assay performed to determine the concentration of the immunoglobulins.

2.2.2) IMMUNOLOCALIZATION OF PAS IV ON TISSUE SECTIONS

Bovine and human sections of different methacorn-fixed tissues were rehydrated by incubating sequentially in xylene, absolute ethanol, 90% and 75% ethanol and distilled water for 3 minutes each. The sections were blocked for nonspecific binding by incubation with 1% goat serum in TBS for 20 minutes. Each section was incubated with affinity purified rabbit anti bovine or anti human antibody in concentrations of 1.2 ug per section. Control sections were incubated with affinity (protein A) purified preimmunized rabbit immunoglobulin. After 1 hour incubation, sections were washed with TBS and incubated with biotinylated goat anti rabbit IgG antibody for 1 hour. The sections were washed and further incubated with a preformed Avidin Biotinylated peroxidase macromolecular Complex (ABC kit) for 1 hour . After further washings, the sections were treated in the dark for 30 minutes with DAB (3, 3'-diamnobenzidine tetrahydrochloride). DAB was prepared by mixing 10 ul of DAB (40 mg/ml), 3 ul of 3% H_2O_2 and 500 ul of TBS. Sections were further treated with hematoxylin (10 minutes) to stain the nuclei and sodium bicarbonate (3 minutes) to intensify the nuclei staining. After dehydration with ethanol the sections were mounted on permount and observed under a microscope (28, 29, 30).

2.2.3) COLLAGEN BINDING TO MILK FAT GLOBULE MEMBRANE

Cyanogen bromide - activated sepharose 4B affinity columns were prepared according to the manufacturer's procedure. Three mg of bovine serum albumin, 3.0 mg of fibrinogen. 3.4 mg of collagen and 3.2 mg of gelatin were used to prepare four different columns. All columns were 2 ml in volume. BCA protein assays were performed for each before

and after immobilization, to calculate the amount of ligand bound on the columns.

The columns were equilibrated with 50 mM Tris buffer pH 7.4 containing 150 mM NaCl and 0.1% Triton X-100 (buffer A). Two ml of solubilized milk fat globule membrane was applied to each affinity column and the columns were washed with 10 ml of buffer A. Five ml of solution containing 100 mM glycine with 1 M NaCl and 0.1% Triton X-100 pH 2.8 was used as eluting buffer. Two ml fractions were collected and 150 ul of 1 M Tris buffer saline with 0.1% Triton X-100 was added to increase the pH to 8.0. All fractions were analyzed on a SDS polyacrylamide gel with solubilized membrane as a marker for PAS IV. Gels were also run for fractions collected while washing the columns. Gels were stained with coomaisse blue to detect the protein bands.

2.2.4) COLLAGEN BINDING TO PURIFIED BOVINE PAS IV

The binding of collagen to purified PAS IV was also examined using enzyme-linked immunosorbent assay studies. Collagen, gelatin, fibrinogen and bovine serum albumin were adsorbed to the bottom of the ELISA plate microtitre wells at a concentration of 2 ug/well in Na_2CO_3 buffer pH 9.6 by incubating overnight. PAS IV was also adsorbed to the wells for use as a positive control. Plates were washed successively with 20 mM Tris buffer containing 150 mM NaCl pH 7.4 (TBS), TBS, 0.1% Triton X-100 and TBS, 1% bovine serum albumin. Nonspecific binding was blocked by incubation with TBS,1% bovine serum albumin for 60 minutes. After washing the wells three times, 2 ug/well of purified PAS IV in TBS, 1% bovine serum albumin pH 7.4 was incubated in the wells for 60 minutes. After additional washings anti bovine PAS IV rabbit antibody (1 ug/well) was added to the wells and incubated for 60 minutes to detect bound PAS IV. Plates were again washed and goat anti

14

rabbit antibody conjugated with horse radish peroxidase was added. After washing the excess enzyme conjugated secondary antibody, the bound enzyme was mixed with ABTS as substrate. Twenty three microliters of ABTS (22 mg/10 ml) was mixed with 7 ul of 0.5 M H_2O_2 and 2.27 ml of citrate buffer pH 4.0 to prepare the substrate. The green color obtained due to the enzyme action was quantified by densitometry.

2.2.5) COMPLEMENT COMPONENT C1Q BINDING TO PURIFIED PAS IV

C1q was adsorbed to the microtitre wells and an enzyme-linked immunosorbent assay was performed as described for collagen.

2.2.6) THROMBOSPONDIN BINDING TO PURIFIED PAS IV

Thrombospondin binding was examined by a slightly different technique. Thrombospondin (4 ug/ml) was adsorbed to the microtitre wells using Na_2CO_3 buffer pH 9.6. Plates were washed with 20 mM Tris buffer pH 7.4 containing 0.15 M or 0.5 M NaCl and 0.05% of detergent Tween 20 (Tris Tween). PAS IV was diluted to a concentration of 4 ug/ml in Tris Tween, 0.5% bovine serum albumin and incubated in the wells for 3 hours at 37°C. After washing with Tris Tween and Tris Tween BSA, plates were incubated with rabbit anti bovine PAS IV antibody at a concentration of 2 ug/ml. The primary antibody was detected by incubation with goat anti- rabbit antibody conjugated with horse radish peroxidase. ABTS (22 mg/10 ml) was used as a substrate. Twenty three microliters of ABTS was mixed with 7 ul of 0.5 M H_2O_2 and 2.27 ml of citrate buffer pH 4.0 to prepare the substrate. The reaction gave a green color which was quantified by densitometry.

CHAPTER 3 : RESULTS

3.1) PURIFICATION OF IMMUNOGLOBULIN

Immediately after loading the whole serum sample, fractions were collected. Fractions were also collected after elution of the immunoglobulins from the column by glycine (pH 2.5). These fractions were mixed with 2-mercaptoethanol and analyzed on sodium dodecyl sulfate polyacrylamide gels. 2-mercaptoethanol cleaves the disulfide bonds between the light and heavy chains of the immunoglobulins. Therefore, the light and heavy chains appear as two different bands on the gel (lane 3, Fig. 1 and lanes 2 and 3, Fig. 2). The molecular weight of the heavy chains is about 50,000 Da and that of the light chains is 25,000 Da (lanes 1 and 3, Fig. 1). The presence of immunoglobulins in the fractions was confirmed by running an immunoglobulin standard (Fig. 2) as well as molecular weight markers (Fig. 1). The fractions showing the presence of immunoglobulins were combined together. Determination of immunoglobulin concentration by the BCA assay technique, gave a protein concentration of 0.5819 mg/ ml of purified preimmune serum from the rabbit immunized with bovine PAS IV. Preimmune serum from the rabbit immunized with human PAS IV contained 0.45 mg/ ml of immunoglobulin after purification.

3.2) IMMUNOLOCALIZATION OF PAS IV

Oxidation of DAB by horse radish peroxidase in presence of H_2O_2, is seen as brown coloration under the microscope. Apical membrane showed specific staining on bovine lactating mammary tissue sections (Fig. 3). Blood capillaries in mammary as well as heart tissue sections stained positive. These sections were used as positive controls for standardizing the procedure. The peripheral epithelial cells of the lung bron-
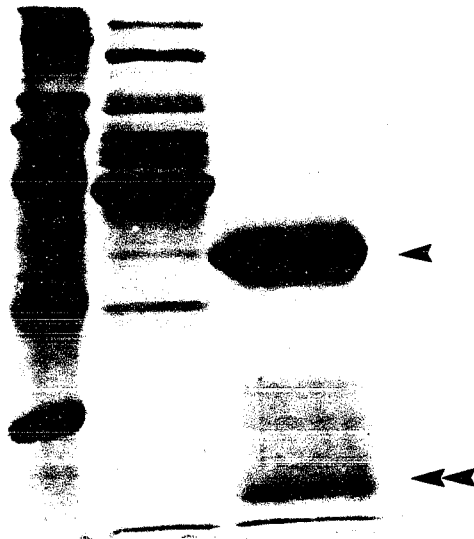
FIGURE 1

SDS PAGE analysis of purified immunoglobulins :

Lane 1, molecular weight markers.

Lane 2, void fraction.

Lane 3, purified immunoglobulins from preimmune serum before immuni-
zation with human PAS IV. Heavy chains (single arrow) show less mobil-
ity due to their high molecular weight (50,000 Da). The light chains
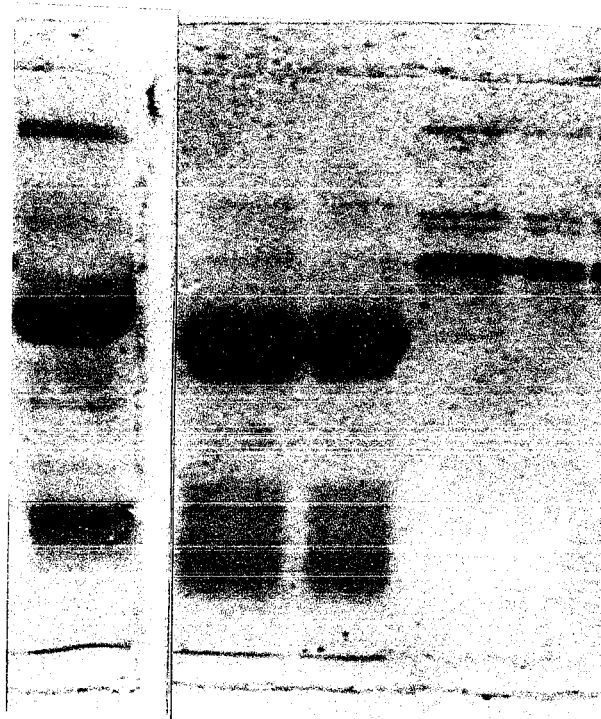(double arrows) form the lower band (molecular weight 25,000 Da)

FIGURE 2

SDS PAGE analysis of purified immunoglobulins :

Lane 1, standard immunoglobulins.

Lane 2 and 3, purified immunoglobulins from preimmune serum. Heavy
and light chains appear as two different bands.

Lane 4 and 5, void fraction after loading the preimmune serum.

chioles were stained positive (Fig. 5). Sections stained with affinity purified control antibody did not show stain of PAS IV on any of the sections (Fig. 4, 7, 10).

Uniform immunostaining was observed on endothelium of normal human breast tissue sections (Fig. 6). The epithelium, however, was heterogenously stained (Fig. 7). Positive staining on blood capillaries was seen. Human liver sections did not show any positive stain. Capillaries on human breast tumor sections stained positive (Fig. 8) but the rest of the section did not show any specific immunostain (Fig. 9). No positive stain was observed on human breast tumor skin sections (not shown).

3.3) COLLAGEN BINDING TO MILK FAT GLOBULE MEMBRANE

Ninety percent of the bovine serum albumin was calculated as bound after a BCA assay was performed on the washings collected during the preparation of the affinity column. Ninety percent of the gelatin, ninety three percent of the fibrinogen and eighty seven percent of the collagen were bound to their respective columns. This means 2.7 mg of bovine serum albumin, 2.8 mg of fibrinogen, 2.9 mg of collagen and 2.8 mg of gelatin were bound to the columns.

Five milliliter fractions were collected immediately after loading of the milk fat globule membrane on each of the columns. These fractions were analyzed on a SDS polyacrylamide gel along with the fractions collected while eluting the column (Fig. 11). Solubilized membrane was included as a standard for comparison of bands. Electrophoretic bands showed the presence of PAS IV in the fractions collected after sample loading (lanes 4 and 7, Fig. 11). Fractions eluted with glycine did not show any bands (lanes 2, 3, 5 and 6, Fig. 11). This means that PAS IV did not bind to any of the columns.
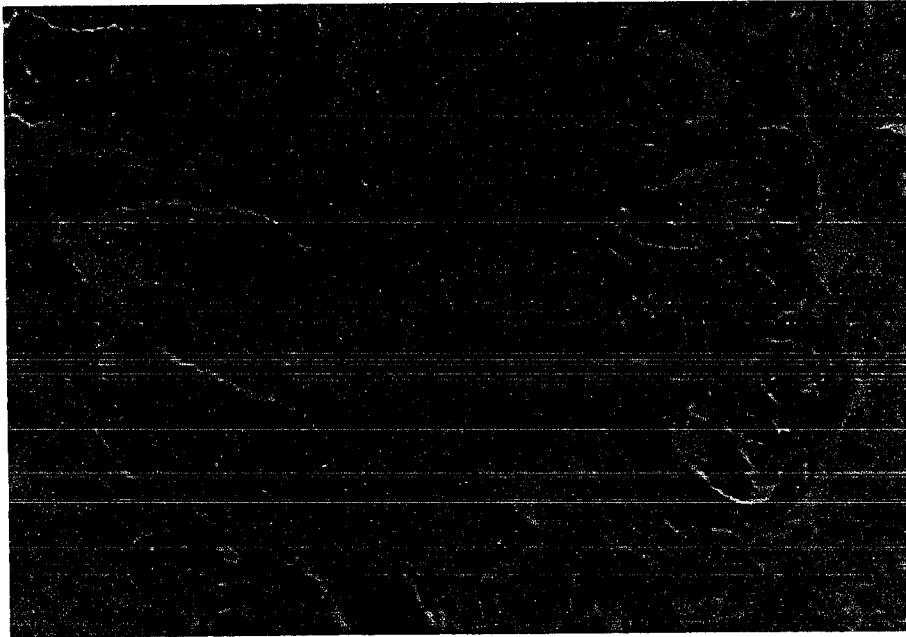
FIGURE 3

Localization of PAS IV in bovine lactating mammary tissue section by ABC technique. Both capillaries (single arrow) and epithelial cells (double arrow) of the alveoli show positive stain of PAS IV. The lumenal contents of the alveoli are darkly stained.

FIGURE 4

Bovine lactating mammary tissue section : affinity purified control anti-
body was used instead of anti bovine PAS IV to detect nonspecific stain.
There is no stain on the capillaries as well as the rest of the tissue.

FIGURE 5

Localization of PAS IV in bovine lung tissue section by ABC technique. The epithelial cells on the periphery of the bronchiole show dark stain of PAS IV (arrow head).
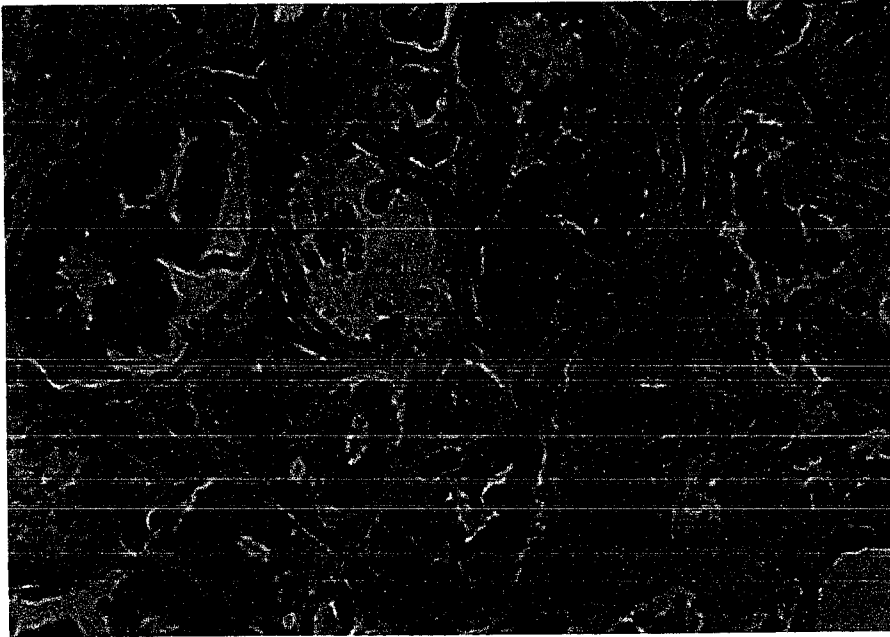
FIGURE 6

Localization of PAS IV in normal human breast tissue section by ABC
technique. The capillaries are stained positive (single arrow). The epithe-
lial cells also show stain of PAS IV (double arrow).
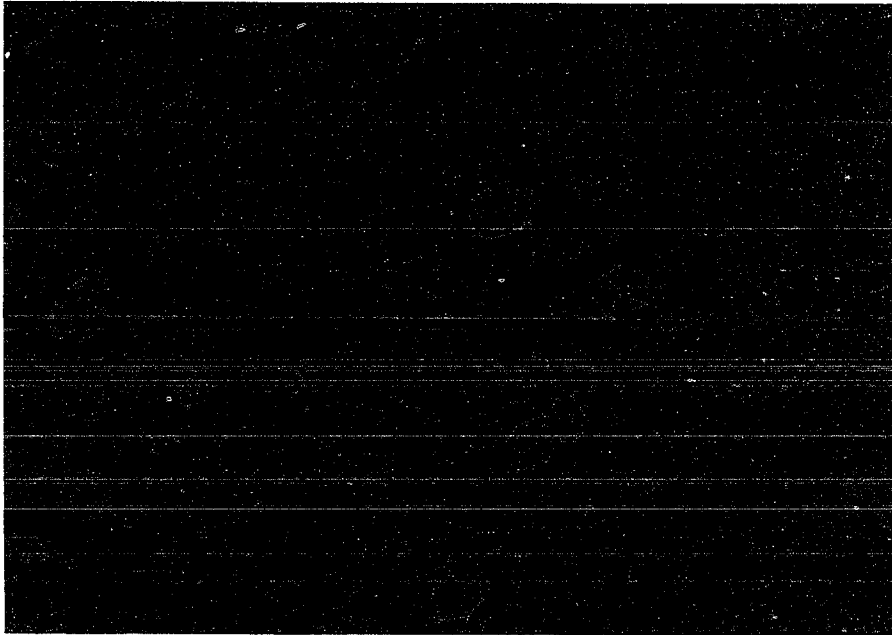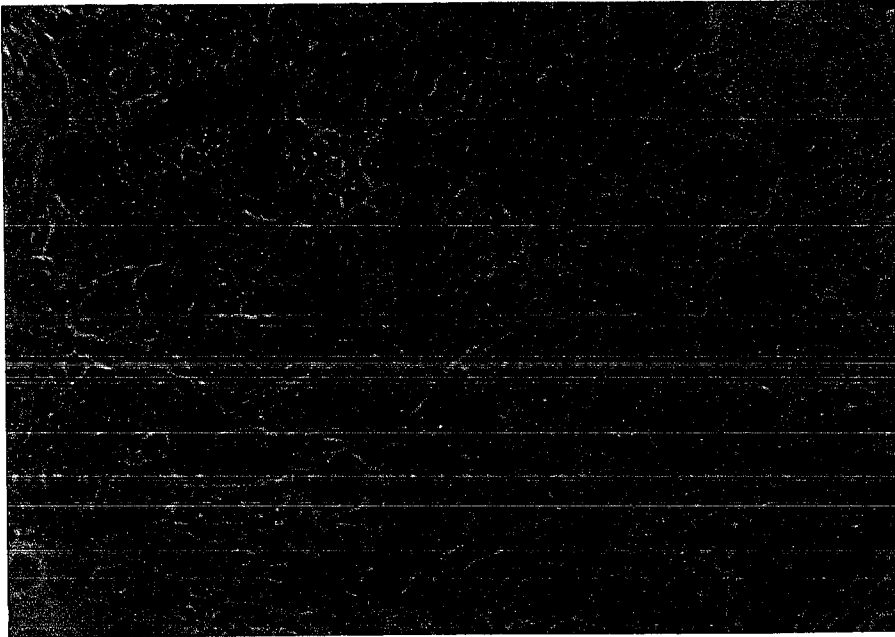
FIGURE 7

Human normal breast tissue section : affinity purified control antibody
was used instead of anti human PAS IV to detect nonspecific stain.
There is no stain on the capillaries as well as the rest of the tissue.
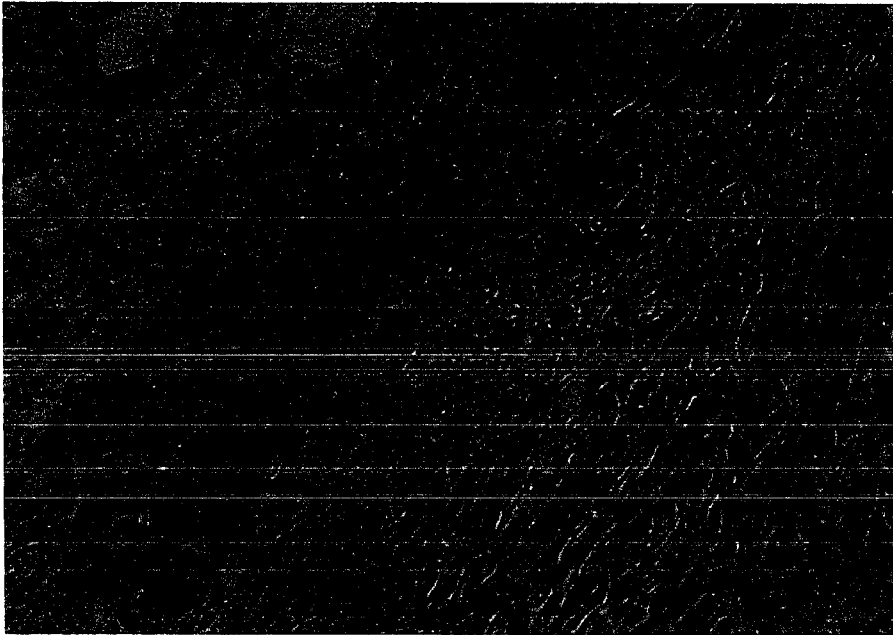
FIGURE 8

Localization of PAS IV in human breast tumor tissue section by ABC
technique. A capillary shows the presence of PAS IV (arrow head).
The rest of the tissue section can be seen in figure 9.

25
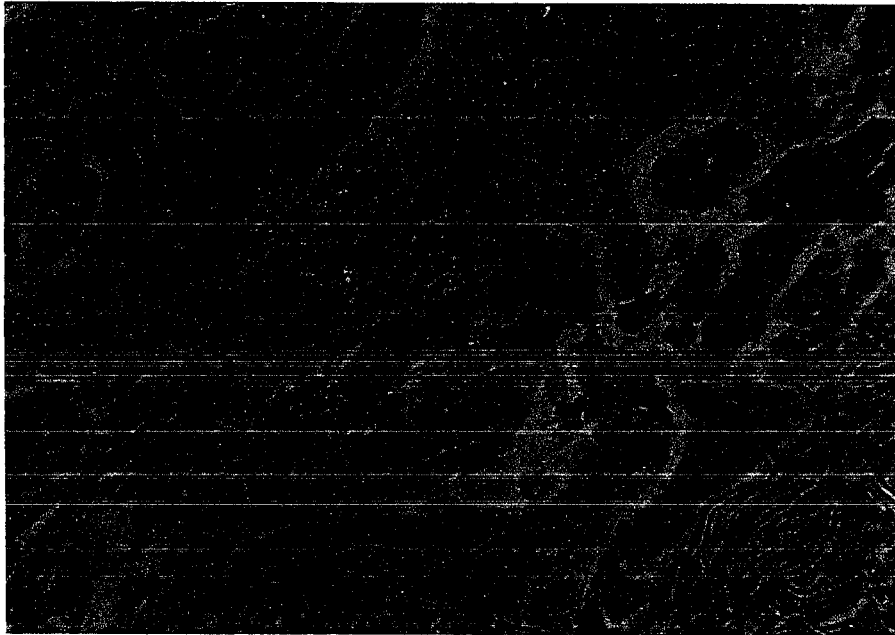
FIGURE 9

Localization of PAS IV in human breast tumor tissue section by ABC technique.
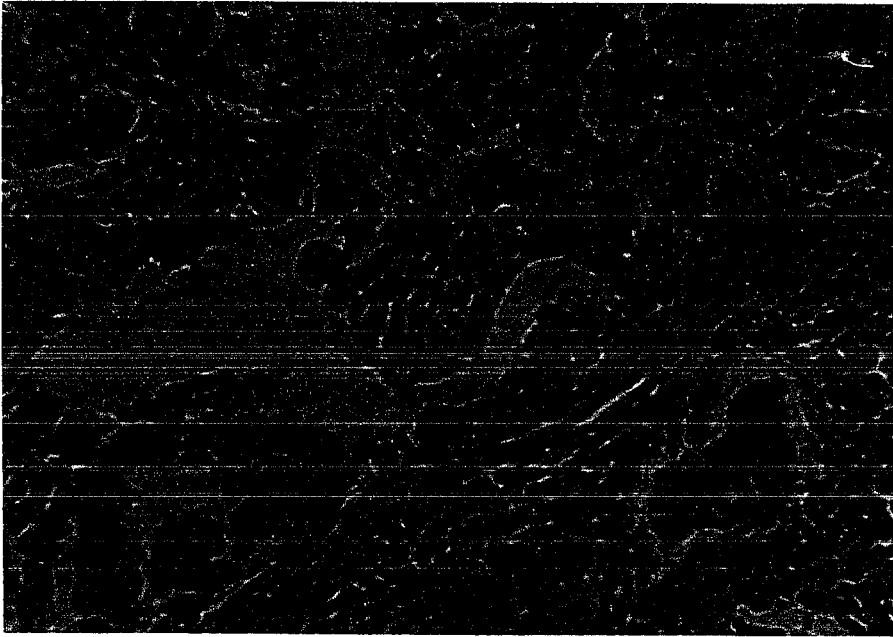
FIGURE 10

Human breast tumor section : Staining with control antibody.
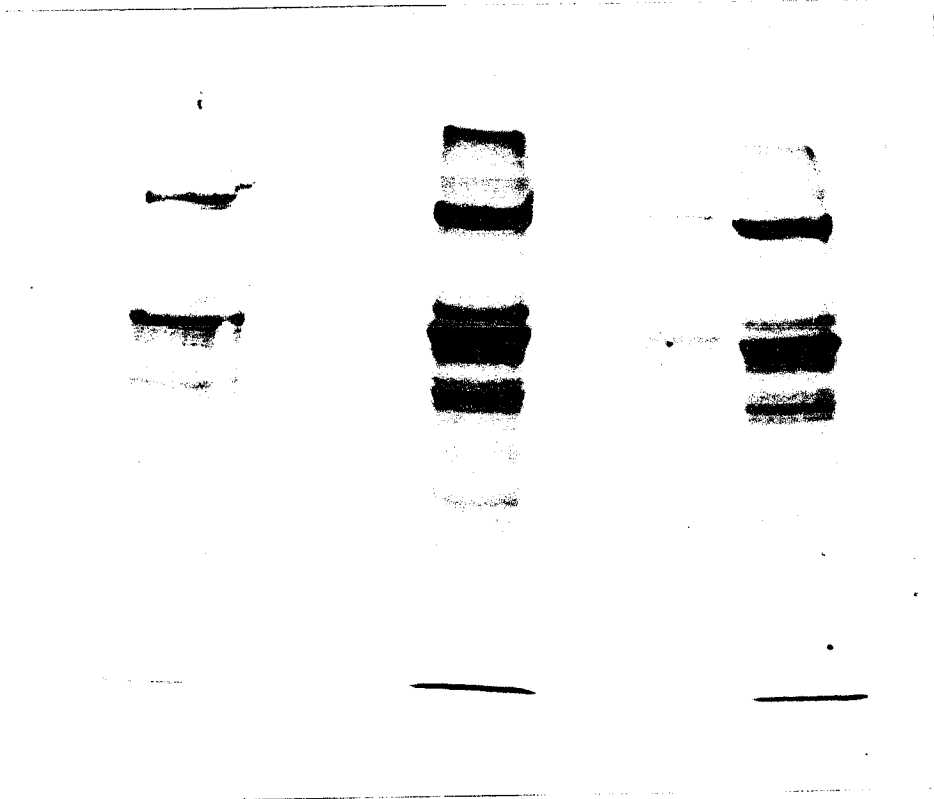
FIGURE 11

SDS PAGE analysis of fractions collected from collagen and gelatin affinity columns :

Lane 1, solubilised membrane.

Lane 2 and 3, eluted fractions from collagen column.

Lane 4, void fraction from collagen column.

Lane 5 and 6, eluted fractions from gelatin column.

Lane 7, void fraction from gelatin column.

3.4) BINDING OF COLLAGEN AND C1Q TO PURIFIED PAS IV

The binding of PAS IV to collagen, C1q and thrombospondin was studied by enzyme linked immunosorbent assay (ELISA). The enzyme used was peroxidase and the substrate was ABTS. Action of peroxidase on ABTS gives a green product. The color density can be measured on an ELISA plate reader. The data obtained for the ELISA assay performed to study PAS IV binding to collagen and C1q are summarized in Tables 2 and 3. PAS IV was coated on the plates to function as positive control. The density values obtained for PAS IV are much higher than that for collagen or C1q incubated with PAS IV. These values are almost as low as for the negative controls, gelatin, bovine serum albumin and fibrinogen.

Both collagen and C1q show similar binding pattern. From the data it may be said that PAS IV does not bind collagen or C1q. PAS IV is known to stick easily on glass and plastic surfaces (32). To determine the extent of nonspecific binding of PAS IV to the plastic of the wells, uncoated plates were also treated in a similar manner as the rest of the wells. Positive coloration was not detected in these wells. Therefore, the error of nonspecific binding was eliminated.

3.5) THROMBOSPONDIN BINDING TO PAS IV

Binding of PAS IV to thrombospondin has been studied (personal communication, L. Leung). We studied the binding by a similar method, except for the different salt concentrations. Prolonged incubation gave us a positive response with the negative controls, gelatin, bovine serum albumin and fibrinogen. The measured density data are summarized in Table 4. From the data it can be seen that the negative controls are more densely colored.

TABLE 2 : COLLAGEN BINDING ASSAY.

Plates coated with	Incubation specifications	Color densities	
		1st set	2nd set
Gelatin	primary antibody	0.096	-0.066, -0.045
Gelatin	control antibody	0.091	-0.029, -0.033
Collagen	primary antibody	0.077	-0.024, -0.037
Collagen	control antibody	0.079	-0.031, -0.027
-	primary antibody	0.098	-0.048, -0.055
-	control antibody	0.125	-0.034, -0.024
PAS IV	control antibody	0.110	-0.033, -0.039
PAS IV	primary antibody	0.442	0.180, 0.165

All wells were incubated with different proteins, in the following order :

Coated well, PAS IV, Primary/Control antibody, Secondary antibody conjugated with peroxidase, substrate.

Uncoated wells were read as blank.

TABLE 3 : C1q BINDING ASSAY.

Plates coated with	Incubation specifications	Color densities
Collagen	primary antibody	-0.009, -0.023
Collagen	control antibody	-0.023, -0.047
Gelatin	primary antibody	-0.050, -0.020
Gelatin	control antibody	-0.028, -0.026
PAS IV	primary antibody	0.145, 0.168
PAS IV	control antibody	-0.018, -0.041
-	primary antibody	-0.003, -0.006
-	control antibody	-0.031, -0.014
C1q	primary antibody	-0.086, -0.021
C1q	control antibody	-0.059, -0.017

All wells were incubated with different proteins, in the following order :

Coated protein, PAS IV, Primary/Control antibody, Secondary antibody conjugated with peroxidase, substrate.

Uncoated wells were read as blank.

TABLE 4 : THROMBOSPONDIN BINDING ASSAY.

Plates coated with	Incubation specifications	Color densities		
		1st set	2nd set	3rd set
TSP	primary antibody, Ca^{+2}	0.007, 0.007	0.013, 0.014	0.031, 0.035
TSP	primary antibody, EDTA	0.004, 0.010	0.035, 0.036	0.059, 0.061
TSP	control antibody	0.002, 0.003	0.004, 0.004	0.003, 0.003
TSP	no secondary antibody	0.002, 0.003	0.003, 0.003	0.001, 0.003
TSP	no primary antibody	0.001, 0.003	0.002, 0.003	0.016, 0.014
TSP	no PAS IV	0.001, 0.004	0.003, 0.004	0.004, 0.013
BSA	primary antibody	0.012, 0.025	0.029, 0.019	0.073, 0.193
Gelatin	primary antibody	0.014, 0.010	0.042, 0.049	0.123, 0.156
Fibrinogen	primary antibody	0.007, 0.009	0.027, 0.025	0.099, 0.121
PAS IV	primary antibody	0.094, 0.140	0.140, 0.132	0.201, 0.213
-	primary antibody	0.004, 0.008	0.021, 0.091	0.014, 0.011

All wells were incubated with different antibodies and PAS IV, in the following order :

Coated well, PAS IV, Primary/Control antibody, Secondary antibody conjugated with peroxidase, substrate.

The 1st set was incubated and washed with buffer containing 0.5 M NaCl, the 2nd set with 0.15M NaCl and the 3rd set with no NaCl.

Uncoated wells were read as blank.

There are two classes of thrombospondin binding sites on platelets. One class is cation independent with low capacity and the other is cation dependent with high capacity. Data from two different references (12, 18) suggest that purified GP IV is responsible for Ca^{+2} dependent binding of thrombospondin to the activated platelet surface. The binding of PAS IV was studied in presence as well as absence of calcium ions. EDTA chelates metal ions like calcium (Ca^{+2}) and these ions would therefore be unavailable for a calcium dependent reaction. Thrombospondin shows more binding in presence of EDTA. The density of the color in presence of calcium is almost half the amount measured in presence of EDTA. The uncoated plates also show positive response to PAS IV.

CHAPTER 4 : DISCUSSION

4.1) IMMUNOLOCALIZATION OF PAS IV ON TISSUE SECTIONS

Success in immunocytochemistry depends on the specificity of the antibody used and the selection of proper tissue processing and immunostaining procedures. Although fluorescence techniques were first developed in early sixties, they did not get wide spread use until a few years later. Immunofluoresence has proved to be an useful tool for the identification of the surface and intracellular lymphocyte markers and immune complexes of glomerular diseases (28). Distribution of PAS IV in various bovine tissues has also been determined by using immunofluorescence microscopy (8). Within the last decade, a new technique has been developed which is rapidly replacing immunofluorescence because of its simplicity and usefulness in fixed tissue sections. This technique, called the ABC technique, uses enzyme labeled antibodies, and the amount of antigen is determined by examining the staining intensity (28).

Avidin is 68,000 molecular weight glycoprotein with an extraordinarily high affinity (10^{15} M^{-1}) for the small molecular weight vitamin, biotin. Because this affinity is one million times higher than that of antibodies for most antigens, the binding of biotin to avidin is essentially irreversible. Most protein molecules (including enzymes and antibodies) can be conjugated with several molecules of biotin. This characteristic provides the potential for macromolecular complexes to be formed between avidin and biotinylated enzymes (30,31).

An immunoperoxidase procedure based on these properties was devised for localizing a variety of histologically significant antigens and other markers. This technique employs unlabeled primary antibody, followed by biotinylated secondary antibody and then a preformed Avidin and Bioti-

34

nylated horseradish peroxidase macromolecular Complex (28, 30, 31). This has been termed the ABC technique.

The ABC technique was used to study the localization of PAS IV on various tissue sections. Previous studies on capillary endothelial cells of bovine lactating mammary tissue (8) have shown the presence of PAS IV. The epithelial cells on the periphery of the lung bronchiole also show positive stain of PAS IV. Normal human breast tissue sections also show presence of PAS IV on capillary endothelial cells. But the heterogeneity of the stain on the epithelial cells of non-lactating, non-differentiating human breast tissue suggests that the amount of PAS IV present on these cells is less than that on the endothelial cells. Positive stain on the capillaries of human breast tumor sections provided us with an internal positive control. The apparent absence of stain on the rest of the tissue may suggest the absence of PAS IV on the cancer cells of the tumor tissues.

Initially, bovine mammary and heart tissue sections were used to standardize the ABC assay procedure. Different buffer systems were used to minimize background stain and maximize the color development of the substrate. Five tumor section slides were stained to confirm our results. At least two slides were stained for all other tissue sections.

The reasons behind the absence of PAS IV on the tumor cells have not been explored. But, it is a known fact that tumor cells behave differently than the normal cells. They are less flattened in culture, as compared to the normal cells, and less dependent on an extracellular matrix for growth (4).

4.2) COLLAGEN BINDING TO MILK FAT GLOBULE MEMBRANE AND PURIFIED PAS IV

Binding of GP IV to collagen has been previously studied (15). Collagen proved to be a ligand for GP IV. In a parallel experiment, we pre-

pared an affinity column with collagen as a ligand. Milk fat globule membrane with a total protein concentration of 20 mg was allowed to bind to 2.9 mg of total collagen on the column. Since PAS IV is 5% of the total protein, there was 1 mg of PAS IV in the membrane preparation. At a protein to ligand ratio of 1:3, PAS IV elutes in the void volume.

Gelatin, bovine serum albumin and fibrinogen were also immobilized on different columns to be used as negative controls. Gelatin is the denatured form of collagen (15). Denatured collagen does not bind GP IV and is without any effect on platelet activation (4, 15). Therefore, gelatin was used as a negative control in our experiments. Bovine serum albumin, gelatin and fibrinogen were used to determine nonspecific binding.

4.3) C1Q BINDING TO PAS IV

As mentioned earlier, GP IV binds C1q. The importance of this binding is not known. C1q is responsible for activating the complement system and thereby is the effector mechanism in the immune defence against infection (26). The results indicate that PAS IV does not bind C1q. Apart from adhesion, other functions for PAS IV have yet to be explored. PAS IV probably has a different role to play on its resident cells. The results suggest a different function for PAS IV.

4.4) THROMBOSPONDIN BINDING TO PAS IV

Binding of PAS IV to thrombospondin has been studied (L. Leung, personal communication). Thrombospondin binding to PAS IV was studied under different salt concentration conditions. The concentrations of salt (NaCl) were 0.15 M, 0.5 M and no salt. Color density results from all different conditions used, indicate a positive response by negative controls. PAS IV is known to be sticky to glassware. Therefore, incubation of PAS IV with the ligand proteins for three hours may result in nonspecif-

ic attachment of PAS IV to the plastic wells. This indicates that thrombospondin may not be binding to PAS IV, and the color development may be nonspecific.

GP IV is a multifunctional membrane protein on platelets which acts as a specific receptor for thrombospondin and mediates platelet aggregation (12). Thrombospondin is released by the alpha granules of the platelets and binds to the receptor on the platelets themselves. In colostrum PAS IV is associated with the milk fat globule (8) while thrombospondin is present in the aqueous phase of the milk (13). There is a possibility that PAS IV does come in contact with thrombospondin in the milk. But it is quite likely that the two proteins do not interact and there presence in the colostrum may entirely be an unrelated fact. This observation can be supported by the fact that the level of thrombospondin drops dramatically as lactation becomes established (13).

All binding studies were performed with purified bovine PAS IV. The thrombospondin used was from human blood platelets. Species specific differences could account for the absence of any specific binding of bovine PAS IV to human thrombospondin.

CHAPTER 5 : CONCLUSIONS

In spite of the similarities between PAS IV and GP IV, there are important differences between the two glycoproteins. The isoelectric point of GP IV is in a pH range of 4.4 to 6.3, while PAS IV has an isoelectric point between pH 7.8 to 8.5. The sialic acid content of PAS IV is about 3.65 moles/mole of protein and that of GP IV is 25.5 moles/mole of protein. All these can be attributed to cell specific differences in the glycosylation of a common polypeptide.

GP IV binds collagen (15) and functions as a receptor for thrombospondin on the platelet surface (12). It also mediates binding of malarial infected red cells to platelets (16) and may act as a receptor for extracellular matrix components. GP IV may represent a new class of adhesive molecules involved in a range of biological functions with different cells and substrates (12).

Based on its similarities to GP IV (Table 1), it has been suggested that PAS IV also functions as a receptor for extracellular matrix components (32). But our studies indicate that PAS IV does not bind collagen and C1q. It is not conclusively proved that PAS IV binds thrombospondin. It does not appear to be a receptor for extracellular matrix components. Even though PAS IV and GP IV have similar physical characteristics, they are localized in different tissues. But their antibodies do cross react. It is possible that inspite of their similarities GP IV and PAS IV may have individual functions in the cells.

REFERENCES

1. Lodish, H. F. and Rothman, J. E., (**1979**) Sci. Am. *240*, 43.

2. Wickner, W., (**1980**) Science, *210*, 861.

3. Molecular Biology of The Cell, Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J. D., Garland Pub. Inc., NY, London.

4. Kleinman, H. K., Klebe, R. J. and Martin, G. R., (**1981**) J. of Cell Bio., *88*, 473.

5. Patton, S. and Keenan, T. W., (**1975**) Biochimica et Biophysica Acta, *415*, 273.

6. Kitchen, B. J., (**1974**) Biochim. et Biophy. Acta, *356*, 257.

7. Mather, I. H. and Keenan, T. W., (**1975**) J. Memb. Bio., *21*, 65.

8. Greenwalt, D. E. and Mather, I. H., (**1985**) J. Cell Biology, *100*, 397.

9. Greenwalt, D. E., Jhonson, V. G. and Mather, I. H., (**1985**), Biochem. J., *228*, 233.

10. Mather I. H., Tamplin, C. B. and Irving, M. G., (**1980**) Eur. J. Biochem., *110*, 327.

11. Jhonson, V. G., Greenwalt, D. E., Mather, I. H., Madara, P. J., (**1988**) Biochem. J., *251*, 507.

12. Asch, A. S., Barnwell, J., Silverstein, R. L. and Nachman, R. L., (**1987**) J. Clinical Invest., *79*, 1054.

13. Dawes, J., Clezardin, P., Ptatt, D. A., (**1987**) Sem. in Thrombo. and Hemostat., *13 (3)*, 378.

14. Wight, T. N., Raugi, G. J., Mumby, S. M., Bornstein, P., (**1985**) J. of Histochem. and Cytochem., *33 (4)*, 295.

15. Tondon, N. N., Kralisz, U., Jamieson, G. A., (**1989**) J. Biol. Chem., *264*, 7576.

16. Ockenhouse, C. F., Tandon, N. N., Magowan, C., Jamieson, G. A., Chulay, J. D., (**1989**) Science, *243*, 1469.

17. Tandon, N. N., Lipsky, R. H., Burgess, W. H., Jamieson, G. A., (**1989**) J. Biol. Chem., *264*, 7570.

18. Kiefer, N., Nurden, A. T., Hasitz, M., Monique, T., Breton-Gorium, J., (**1988**) Biochem. et Biophy. Acta, *967*, 408.

19. Miller, W. R., Dawes, J., Brown, K., (**1985**) Thrombo. Haemost., *54*, 64.

20. Borchert, M., Lanket-Bultgereit, B., Mauch, C., Pfaffle, M., Deutz-mann, R., Von der Mark, K., Kreig, T., (**1989**) J. of Invest. Dermat., *92 (3)*, 406.

21. McCall, M. N., Easterbrook-Smith, S. B., (**1989**) Biochem. J., *257*, 845.

22. Tandon, N. N., Hines, A. and Jamieson. G. A., (**1985**) Blood. *66*, 1148.

23. Muggli, R. and Baumgartner, H. R., (**1973**) Thrombo. Res., *3*, 715.

24. Jaffe, R. and Deykin, D., (**1974**) J. of Clin. Invest., *53*, 875.

25. Oquenda, P., Hundt, E.; Lawler, J. and Seed, B., (**1989**) Cell, *58*, 95.

26. Porter, R. R. and Reid, K. B. M., (**1978**) Nature, *275*, 699.

27. Larsson, A. and Sjoquist, J., (**1989**) J. of Immuno. Methods, *119*, 103.

28. Hsu, S. M., Enzyme Mediated Immunoassay, 467; Edrs; Ngo, T. T. and Lenhoff, H. M., Univ of California, Irvine.

29. Ching, K., Feng-Chen, Chen, B., Liu, Z., Ng, A. K., (**1986**) J. of His-tochem. and Cytochem., *34 (11)*, 1495.

30. Hsu, S. M., Raine, L., Fanger, H., (1981) J. of Histochem. and Cytochem., *29*, 577.

31. Hsu, S. M., Raine, L. and Fanger, H., (1981) Am. J. Clin. Pathol., *75*, 734.

32. Greenwalt, D. E., So, O. Y., Watt, K. W. K. and Jiwani, N. (1990) J. Biol. Chem., in press.

33. Compton, B. J., Lewis, M., Whigham, F., Gerald, J. S. and Countryman, G. E. (1989) Analytical Chem., *61*, 1314.

34. O'Farrell, P. H. (1975) J. Biol. Chem., *250 (10)*, 4007.